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Page 108, line 22, "between 0.05 and 0.0100 per cent" should read "between 0.05 and 0.100 per cent."

Page 217, heading, "The Citrus-Root Nematode Tylenchus Semipenetrans" should read "The Citrus-Root Nematode Tylenchulus Semipenetrans."

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FLAVOR OF ROQUEFORT CHEESE

By JAMES N. CURRIE,[1]
Chemist, Dairy Division, Bureau of Animal Industry

INTRODUCTION

Every cheese connoisseur is familiar with the peculiar peppery or burning effect of well-ripened Roquefort cheese on the organs of taste. This effect is so characteristic of this variety of cheese that its quality is commonly expressed in terms of its "hotness." The purpose of this investigation was to identify and to explain the occurrence in the cheese of any substances which contribute to this particular flavor.

WORK OF OTHER INVESTIGATORS

Weigmann (16, p. 187)[2], without citing the source of his information, says:

> It has long been known that the characteristic rancid, sharp taste of French Roquefort, English Stilton, and Italian Gorgonzola cheeses is caused by the green Penicillium. This characteristic taste is first observed after the spores and spore bearers have formed and has therefore been ascribed to them.

If a chemical substance which imparts to the cheese this burning taste were elaborated in the spores and spore bearers, the mycelium of the mold grown upon artificial media should show this taste in a marked degree at the spore-bearing stage. No taste, however, can be detected at any stage, except a slight bitterness.

Jensen [3] concluded that the chief constituent of the aroma of Roquefort cheese was the "very sharp-tasting ethyl butyrate," but offered no data to show that this ester was actually present. He apparently identified it only by the odor.

Esters are so commonly known as the flavoring substances of fruits that it is quite natural to ascribe to them a part in the production of flavor in other foodstuffs. In fact, Suzuki, Hastings, and Hart (13, p.

[1] The writer takes pleasure in acknowledging his indebtedness to Dr. Charles Thom, who has kindly provided him with all the pure cultures of *Penicillium roqueforti* used in this investigation.

[2] Bibliographic citations in parentheses refer to "Literature cited," p. 13-14.

456), found small quantities of alcohols and esters in the neutral "flavor solution" obtained from Cheddar cheese.

The neutral flavor solution from 750 grams of a ripe Roquefort cheese was studied, using the method outlined by the above-mentioned investigators. The acids in ester combination in this large mass of cheese totaled only 0.44 decinormal c. c. It is hardly possible to identify accurately the acids of a mixture so small in quantity. The proportional numbers obtained by a Duclaux distillation approximated the constants for acetic acid. The odor of Roquefort cheese suggests ethyl acetate, and this ester may be partially responsible for the typical aroma, but it is doubtful whether the quantity is large enough to materially influence the taste.

As early as 1877, Nencki (10, p. 1033), attempted to isolate the substance giving to Roquefort cheese its piquant taste. He acidified about one pound of cheese with sulphuric acid and distilled it with steam. After filtering the distillate he neutralized it with sodium hydroxid and extracted it with ether. After evaporation the ether extract left a "very volatile, slightly yellow-colored oil of sharp burning taste, neutral reaction, and characteristic moldy odor." The quantity of this oil was not sufficient for identification.

Repeated attempts with the ripest cheese procurable were made to duplicate the results of Nencki. Very small quantities of a gummy white mass were obtained, which consisted chiefly of the sodium salts of butyric and caproic acids. Although the oil described by Nencki was not found by following his procedure, it was observed that globules of an insoluble oil floated upon the surface of the distillate. This oil, which Nencki apparently filtered off and discarded, was identified as a mixture of the volatile and soluble acids of milk fat. The amount of these insoluble acids increased with the ripeness of the cheese distilled and appeared to be a normal product of the curing process. Since these acids possessed a peppery taste and the cheese mass after distillation was almost tasteless, a detailed study of their origin and relation to the flavor of the cheese was made.

EXPERIMENTAL METHODS EMPLOYED

Typical Roquefort cheeses of various stages of ripeness were purchased in the retail markets and used in these experiments. In general, the method of study outlined by Jensen was followed.

After scraping off any slime on the surface of the cheese, a wedge-shaped section extending from the periphery to the center was cut out, minced, and thoroughly mixed. A sample of 50 to 150 grams, depending on the ripeness of the cheese, was weighed, rubbed to a smooth cream with warm water in a mortar, and rinsed into a 500 c. c. Kjeldahl flask. The suspension was made up to a volume of 250 c. c. of dilute sulphuric acid added in slight excess, as indicated by a blue color with Congo red, and

distilled with steam until 1,000 c. c. of distillate passed over. During the distillation a small flame was kept under the Kjeldahl flask and regulated so that the volume of the suspension remained nearly constant.

The fatty acids which separated from the distillate were filtered off, dissolved in alcohol, titrated with decinormal barium hydroxid, and reported as insoluble acids.

The filtrate was neutralized with decinormal barium hydroxid and evaporated to about 100 c. c. This solution was then partially decomposed with normal sulphuric acid and the liberated acids distilled out. The distillate was designated "Fraction I." The residue was again made up to 100 c. c., partially decomposed with normal sulphuric acid, and distilled, giving Fraction II. By repeating this process of partial decomposition followed by distillation the soluble acid portion was divided into several fractions containing about equivalent quantities of acid. The acids of greater molecular weight occur in the first fractions because of their weaker chemical affinity and more rapid rate of volatilization.

A QUALITATIVE STUDY OF THE VOLATILE ACIDS

The distillate of a well-ripened cheese was divided into five fractions. Fraction I consisted of the insoluble acids adhering to the condenser after rinsing with cold water. The remainder of the insoluble acids constituted Fraction II. The soluble acids were divided into Fractions III, IV, and V by the process of partial decomposition of the barium salts with normal sulphuric acid followed by distillation, as previously described.

The barium salts were prepared by titrating with decinormal barium hydroxid, dried to constant weight at 120° C., moistened with dilute sulphuric acid, ignited, and weighed as barium sulphate. The results are detailed in Table I.

TABLE I.—*The barium salts of the volatile acids of Roquefort cheese.*

Fraction No.	Decinormal equivalent.	Percentage of barium sulphate found from barium salts.		
		Salt.	Actual.	Theoretical.
	C. c.		*Per cent.*	*Per cent.*
I	2.25	Barium caprate.....	45.53	48.64
II	5.80	Barium caprylate...	47.18	55.08
III	17.32	Barium caproate....	64.67	63.48
IV	17.36	Barium butyrate....	76.07	74.91
V	18.58	Barium acetate.....	88.65	91.37

A study of the above results shows that the distillate from this variety of cheese is essentially a mixture of the volatile acids of milk fat. Capric acid is the chief constituent of the insoluble-acid portion. The fact that no fraction shows the presence of an acid of a molecular weight between capric and caproic indicates that the quantity of caprylic acid is small.

QUANTITATIVE ESTIMATION OF THE VOLATILE ACIDS

The Duclaux method of fractional distillation was employed for the quantitative estimation of the volatile acids.

In order to illustrate fully this method, complete data for one cheese will be given. The soluble-acid portion of the distillate from 150 grams of this cheese required for neutralization 57.20 c. c. of decinormal barium hydroxid, and the insoluble-acid portion, 12.15 c. c.

The soluble-acid portion was divided into four fractions, which were distilled in the order of numbering. The remaining 10 c. c. of one fraction was always added to the succeeding fraction before making up to 110 c. c. Therefore, the only acids not taken into account would be in the last 10 c. c. of the final fraction. These can be readily calculated, since the 100 c. c. of distillate collected contained all of the caproic, 97.5 per cent of the butyric, and 80 per cent of the acetic acid.

The data for the analysis of the soluble-acid portion are given in Table II. A represents the titer of each successive 10 c. c. portion of the distillate; B, the sum of these titers; C, the percentage of B in terms of the acidity of 100 c. c. of distillate, and D, the corresponding figures calculated from the Duclaux constants for the combination of acids indicated.

TABLE II.—*Data for the quantitative estimation of the soluble acids in Roquefort cheese.*

FRACTION I.[1]

Item.	10 c. c.	20 c. c.	30 c. c.	40 c. c.	50 c. c.	60 c. c.	70 c. c.	80 c. c.	90 c. c.	100 c. c.
A....	3.01	2.29	1.71	1.04	0.71	0.49	0.34	0.27	0.18	0.14
B....	3.01	5.30	7.01	8.05	8.76	9.25	9.59	9.86	10.04	10.18
C....	29.57	52.06	68.86	79.08	86.05	90.86	94.20	96.86	98.62	100.00
D....	29.05	49.73	67.66	78.72	86.37	91.64	94.42	96.75	98.76	100.00

FRACTION II.[2]

A....	3.10	2.20	1.64	1.12	.85	.65	.47	.36	.27	.19
B....	3.10	5.30	6.94	8.06	8.91	9.56	10.03	10.39	10.66	10.85
C....	28.57	48.85	63.96	74.29	82.12	88.11	92.44	95.76	98.25	100.00
D....	26.18	45.70	62.62	74.04	82.44	88.68	92.44	95.69	98.24	100.00

FRACTION III.[3]

A....	2.88	2.22	1.71	1.32	1.02	.82	.65	.51	.37	.30
B....	2.88	5.10	6.81	8.13	9.15	9.97	10.62	11.13	11.50	11.80
C....	24.41	43.22	57.71	68.90	77.54	84.49	90.00	94.32	97.45	100.00
D....	23.45	41.64	57.49	69.00	77.87	84.85	89.61	93.83	97.25	100.00

[1] 72 parts of caproic and 28 parts of butyric acid; 7.33 c. c. of caproic and 2.85 c. c. of butyric acid.

[2] 54 parts of caproic and 46 parts butyric acid; 5.86 c. c. caproic and 4.99 c. c. butyric acid.

[3] 40 parts caproic, 55 parts of butyric, and 5 parts of acetic acid; 4.72 c. c. of caproic, 6.49 c. c. butyric, 0.59 c. c. acetic acid.

TABLE II.—*Data for the quantitative estimation of the soluble acids in Roquefort cheese*—Continued.

FRACTION IV.[1]

Item.	10 c. c.	20 c. c.	30 c. c.	40 c. c.	50 c. c.	60 c. c.	70 c. c.	80 c. c.	90 c. c.	100 c. c.
A....	3.56	3.07	2.67	2.32	2.10	1.79	1.59	1.43	1.31	1.20
B....	3.56	6.63	9.30	11.62	13.72	15.51	17.10	18.53	19.84	21.04
C....	16.92	31.51	44.20	55.23	65.21	73.71	81.46	88.07	94.29	100.00
D....	16.75	31.43	44.52	55.88	65.67	74.24	81.38	88.15	94.21	100.00

[1] 10 parts of caproic, 66 parts of butyric, and 24 parts of acetic acid; 2.10 c. c. of caproic, 13.89 c. c. of butyric, and 5.05 c. c. of acetic acid.

Calculated for 110 c. c.: 2.10 c. c. of caproic, 14.25 c. c. of butyric, and 6.31 c. c. of acetic acid.

Totals for 150 grams of cheese: 20.01 c. c. of caproic, 28.58 c. c. of butyric, and 6.90 c. c. of acetic acid.

Calculated for 100 grams of cheese: 13.34 c. c. of caproic, 19.05 c. c. of butyric, and 4.60 c. c. of acetic acid.

Complete analyses were not made of all the cheeses investigated, and for uniformity all results have been calculated to 100 grams of cheese. Roquefort cheese is very uniform in composition, and the significance of the results would in nowise be altered by calculating to dry matter. Dox (5, p. 239) made careful analyses of eight brands of Roquefort cheese. His results are summarized in Table III.

TABLE III.—*Summary of the analyses of eight brands of Roquefort cheese.*

Degree.	Water.	Fat.	Protein.	Ash.	Salt.
	Per cent.	*Per cent.*	*Per cent.*	*Per cent.*	*Per cent.*
Maximum	40.10	33.53	23.25	6.81	4.50
Minimum	37.49	31.50	19.94	5.48	3.64
Average	38.61	32.24	21.62	6.19	4.18

It is to be noted that the sum of the acids recovered by distillation is only 55.49 c. c., or 1.71 c. c. less than the original acidity. A loss of about this magnitude was always experienced. Part of this loss may be due to carbon dioxid in the original distillate which is not completely redissolved in the process of repeated distillation; another part of it is probably due to decomposition by heat, as observed by Browne (3, p. 819). There is also a slight loss of acid during evaporation, for the salts of the weak organic acids are appreciably hydrolyzed, and an odor of the acids can be readily detected above a hot solution neutral to phenolphthalein.

Inspection of these data indicates that there was probably a small quantity of caprylic acid in the first fractions and a small amount of formic acid in the last fraction. These acids, if present at all, were in too small quantities to be determined accurately by this method and were not entered in the calculations.

The foregoing results are regarded as typical of a well-ripened Roquefort cheese. It is to be expected that a mild cheese will have a lower volatile acid number and a very ripe cheese a higher one. Analyses of the acid distillates of cheeses of differing degrees of ripeness have been made and are summarized in Table IV.

TABLE IV.—*Volatile acids in 100 grams of cheese.*

[Acidity in decinormal c. c.]

No.	Condition of cheese.	Total volatile acids.	Insoluble acids.	Soluble acids.	Caproic acid.	Butyric acid.	Acetic acid.	Acid number for 10 grams of fat.
1	Slightly ripened.....	15.07	2.90	12.17	6.85	4.14	1.18	.....
2	Well ripened...	45.09	8.10	36.99	13.34	19.05	4.60	56.4
3	Overripened....	102.23	29.30	72.93	30.38	36.30	6.25	152.6

For the determination of the insoluble-acid number of 10 grams of fat reported in the above table, the procedure of Schmid-Bondzynski [1] was followed. The cheese was digested in warm hydrochloric acid of a specific gravity of 1.125 until the fat separated. It was then washed free from hydrochloric acid, was dried and filtered clear. A sample of the fat was weighed out, dissolved in 95 per cent alcohol, and titrated with decinormal sodium hydroxid. The result was calculated to 10 grams of fat.

In order to follow up the increase in acidity of the distillate during progressive ripening, about 1 pound of cheese was minced and placed in a large bottle. A 50-gram sample was immediately weighed out and distilled. The cheese was kept at about 23° C., and on the dates given in Table V the mass was thoroughly mixed and other 50-gram samples were taken and distilled. During this time the cheese developed no abnormal flavors.

TABLE V.—*Volatile acids in 100 grams of cheese.*

[Acidity in decinormal c. c.]

Date.	Total volatile acids.	Insoluble acids.	Soluble acids.	Caproic acid.	Butyric acid.	Acetic acid.
Mar. 16.....	44.52	8.64	35.88	12.16	21.56	2.16
Mar. 18.....	50.02	11.30	38.72	12.96	21.12	4.64
Mar. 22.....	79.98	22.20	57.78	20.04	32.64	5.10
Apr. 1.....	122.60	33.20	89.40	38.88	46.20	4.52

[1] See Barthel, Chr. (2, p. 187).

From the data in Tables IV and V and from observations on numerous other cheeses, which have been distilled without making complete analyses of the distillates, we conclude that a well-flavored Roquefort cheese will have a distillation number (c. c. of decinormal alkali to neutralize the distillate from 100 grams of cheese) of 30 to 60; one showing a slight growth of mold and very little flavor will have a distillation number less than 30; and a cheese thoroughly permeated with mold and highly flavored will have a distillation number above 60, and in extreme cases this number may even exceed 100.

RELATION OF THE VOLATILE ACIDS TO FLAVOR

In discussions of the properties of this homologous series of saturated fatty acids, a fact of very peculiar interest is commonly overlooked—namely, the pronounced changes in taste with increasing molecular weight. The lower members are distinctly sour and in dilute solutions can not be distinguished from the mineral acids by their taste. This sourness diminishes with increasing carbon content and can not be detected at all in those containing more than 7 carbon atoms. Valeric acid, with 5 carbon atoms, has not only a sour taste but also a distinct peppery effect. This peppery effect increases with increasing molecular weight and is very pronounced in caprylic and capric acids. The higher acids of this series, containing 12 or more carbon atoms, have no well-defined taste. Thus, it is seen that the group of acids containing from 5 to 10 carbon atoms, which includes the three volatile acids of milk fat—caproic, caprylic, and capric—is characterized by this burning or peppery effect on the organs of taste.

Volatile acids have been vaguely connected with the aroma and flavor of dairy products by numerous investigators, but to our knowledge the peculiar peppery effect of the green-mold cheeses has never been specifically attributed to this group of volatile, difficultly soluble acids of milk fat. Duclaux (7, p. 288), in a discussion of Cantal cheese, states that "The sharp taste of old cheese is in large part due to the fixed fatty acids and their salts, which have a burning effect on the palate and tongue." Just what Duclaux meant by "fixed" acids is not quite plain, but certainly his observation is applicable only to the volatile acids. We believe that the unmistakable similarity between the effect on the gustatory nerves of well-ripened Roquefort cheese and these volatile acids entirely justifies the conclusion that the peppery taste of the cheese is due to the accumulation of these acids and their readily hydrolyzable salts.

This explanation is not offered as a solution of the entire problem of the flavor of Roquefort cheese. This is rather to be interpreted as an endeavor to identify a single prominent component of the flavor and to explain its occurrence in the cheese.

FORM IN WHICH VOLATILE ACIDS OCCUR

Another point concerning the relation of these acids to the flavor of cheese remains to be considered—that is, the form in which they are combined. It is not to be expected that the acids are all in the free state, for the proteolysis taking place during the ripening process gives rise to ammonia and possibly other basic substances. The exact course of this proteolysis has not been determined. In a ripe Roquefort cheese examined by Jensen 52.50 per cent of the total nitrogen was water-soluble, 23.64 per cent was precipitated by phosphotungstic acid, and 4.99 per cent was in the form of ammonia. This is in accord with unpublished data of Dox,[1] who made a separation of the different classes of nitrogenous products by the method of Van Slyke and Hart (15, p. 150). For a prime Roquefort he gives the distribution of nitrogen as follows: In caseoses, 10.7; in peptones, 8.6; in amino acids, 29.1; in ammonia, 6.1; insoluble, 45.5. Dox (6, p. 423) has, furthermore, identified tyrosin among the amino acids. The work of these investigators indicates that probably all of the hydrolytic cleavage products of paracasein are to be expected in a ripe Roquefort cheese.

Every Roquefort cheese examined in the laboratory of the Dairy Division has been decidedly acid to both litmus and phenolphthalein. The exact amount of acidity is difficult to measure, for such a complex mixture of weak acids and bases can not be accurately titrated. Ten grams of a well-ripened cheese having a distillation number of 43.9 were extracted three times with 50 c. c. of 95 per cent alcohol. This alcoholic extract required 57.0 c. c. of decinormal alkali for neutralization. Data given by Jensen show that the ammonia is not equivalent to the acidity calculated from the distillation number and the acid number of the fat. This does not take into account the acidity of the paracasein and amino acids.

It is also to be remembered that the ammonium salts of the weak organic acids are so strongly hydrolyzed in an aqueous solution that they can not be crystallized from this solvent. However, they can be readily prepared by passing dry ammonia into a benzene solution of the acids. Ammonium caproate, caprylate, and caprate have been prepared in this manner. They are white needlelike crystals which readily give off the odor of the respective acids when exposed to the moisture of the air. The peppery effect of these salts when placed upon the tongue is quite similar to the effect of the free acids, but is less intense.

From these considerations of the basic and acid substances of the cheese it is apparent that caproic, caprylic, and capric acids exist, both free and combined, and those combined are in such weak form of combination that their characteristic taste is not obscured.

[1] Dox, A. W. Records of the Storrs Agricultural Experiment Station.

ORIGIN OF THE VOLATILE ACIDS OF ROQUEFORT CHEESE

Neuberg and Rosenberg (12, p. 178) have shown that the volatile fatty acids may arise from the putrefaction of casein. From 1 kilogram of casein they isolated 40 grams of butyric acid, 95 grams of caproic acid, 5.0 grams of capric acid, and a considerable quantity of valeric acid. More recently Neuberg (11, p. 501) has shown that putrefactive bacteria produce normal valeric acid from prolin and active valeric acid and active caproic acid from isoleucin. Valeric acid has long been considered as a putrefactive product, and its occurrence in Limburger cheese accounted for in this manner.

The flavor of Roquefort cheese gives no suggestion whatever of the products accompanying putrefaction. Since the volatile acids include those represented in milk fat from lauric to butyric and in about the same proportions as in milk fat, we are led to conclude that they arise from a hydrolysis of the fat.

There is no conclusive evidence that the glycerids of fat are unequally attacked by lipolytic enzyms, and if the volatile acids result from a hydrolysis of the fat the insoluble acid number of the cheese fat should be about proportional to the volatile acid number of the cheese. This is shown to be the case in Table IV.

The biological significance of the small quantity of acetic and possibly formic acid present is not clear. However, these may result from a fermentation of the carbohydrates in the early stages of ripening or may be a product of the partial oxidation of higher acids or glycerin by the mold.

CULTURAL STUDIES OF PENICILLIUM ROQUEFORTI

No biological study ever reported has shown that any other organism than *Penicillium roqueforti* is essential to the proper ripening of this variety of cheese. Some of the proteolysis is to be attributed to the rennet, as is the case with all renneted cheeses, and the bacterial flora common to milk is responsible for the development of acidity in the curd during the early stages of ripening. The hydrolysis of the fat can hardly be ascribed to either of these agencies. So to obtain more definite information concerning the action of *P. roqueforti* on butter fat, various cultural studies have been made.

Czapek's solution, with such modifications as will be specified, was sterilized with steam and used for all cultures on liquid media.

Penicillium roqueforti will grow upon such a solution of inorganic salts as this medium contains when cane sugar is wholly replaced by pure butter fat, tributyrin, ethyl butyrate, glycerin, butyric acid, or ammonium butyrate. Therefore the mold not only has the ability to hydrolyze simple esters and triglycerids but also to utilize their constituents as sources of carbon. This would presuppose the presence of a lipolytic enzym in the organism. Generally, the action of an enzym-containing extract of an organism is so much feebler than the action of the living

organism that the latter method of study has been adopted as affording a truer picture of what the mold actually accomplishes in a ripening cheese.

Penicillium roqueforti was grown upon 100 c. c. of Czapek's solution in which cane sugar was replaced by 3 grams of fresh, filtered milk fat. After 50 days at about 23° C. dilute sulphuric acid was added to the culture until a blue color with Congo red was produced. The solids consisting of fat and mold mycelium were filtered off, washed with hot water, dried at 100° C., and extracted over anhydrous copper sulphate with ether in a Soxhlet extractor. The fat of the ether extract was examined and gave the results shown in Table VI.

TABLE VI.—*The effect of the growth of mold on the composition of milk fat.*

Condition.	Age.	Reichert-Meissl number for 2.5 grams.	Acid number for 10 grams in decinormal c. c.	Molecular weight of insoluble nonvolatile acids.
	Days.			
Uninoculated	50	15.87	3.20	267.6
Inoculated	50	[1] 5.66	157.40	271.7

[1] Determination made on 1.8688 grams and calculated for 2.5 grams.

The above data show that the fat of the uninoculated control gave constants typical of normal milk fat and consequently had undergone no changes, while the fat upon which *Penicillium roqueforti* had grown had been about two-thirds hydrolyzed. The filtrate from the culture contained no volatile acids, which would indicate that the soluble acids of the decomposed fat are consumed completely by the mold, while the insoluble acids are much less readily consumed. That other molds attack the fatty acids of low molecular weight more readily than those of higher molecular weight has been demonstrated by Laxa (9, p. 119). This explains the high acid number of the ether extract, for it is evident that if all the acids resulting from the hydrolysis of the fat were consumed, the ether extract of the mold culture would have been nearly neutral and would have shown the Reichert-Meissl number of normal milk fat.

The conditions of food supply maintained in a Roquefort cheese would be more nearly simulated by growing the mold upon fresh curd than upon solids suspended in a liquid medium. Table VI shows the result of the action of the mold on the milk fat in such a culture. To 50 grams of fresh curd containing about 50 per cent of water 2 grams of sodium chlorid were added. (Roquefort cheese contains about 4 to 5 per cent of sodium chlorid.) The curd was sterilized and inoculated with *Penicillium roqueforti* and kept at about 23° C. On the dates designated the fat was separated by the Schmid-Bondzynski method and was examined. See Table VII.

TABLE VII.—*The effect of Penicillium roqueforti on the fat of fresh curd.*

Condition.	Age.	Reichert-Meissl number for 2.5 grams.	Decinormal acid number for 10 grams.	Molecular weight of the insoluble fatty acids.
	Days.			
Uninoculated control	36	16.05	2.3	269.3
Inoculated	36	12.74	66.7	271.3
Do	85	7.87	163.7	269.4

As in the previous culture on Czapek's solution, the fat showed decided decomposition, but there was only a meager accumulation of soluble and volatile acids. A culture at the age of 45 days contained only 0.75 decinormal c. c. of soluble acids in 500 c. c. of distillate. A culture similarly grown but in the presence of *Bacillus lactis acidi* contained only 0.80 decinormal c. c. of soluble acids in a like volume of distillate. The acid number of the fat in both cultures showed that more than one-half of it had been hydrolyzed.

Just why these acids accumulate in a cheese but not in a culture grown upon green curd is not obvious. The growth of mold in the crevices of the cheese is always very scant in comparison with the growth of a culture having an unlimited supply of oxygen (Thom and Currie, 14, p. 249). The gas within the cheese frequently contains less than 5 per cent by volume of oxygen. This very limited supply of oxygen may hinder the metabolic functions of the mold and prevent the complete oxidation of butyric and caproic acids.

Another explanation, and to the writer this appears the more probable one, is to be found in the presence of a water-soluble enzym which diffuses beyond the feeding zone of the mold. An examination of a Roquefort cheese will always show that the central portion is much more open in texture and more thoroughly permeated with mold than the outer portion. In fact, a layer about 2 cm. in thickness next to the rind is almost free from mold. This portion, although not so highly flavored as the more moldy portion, is always sufficiently ripened to be entirely palatable. The inner portion of a very ripe cheese gave a distillation number of 88.3 and this outer portion, 60. This thorough ripening in portions of the cheese where only small and scattered pockets of the mold are visible is apparently due to water-soluble enzyms, among which is an active lipase.

ENZYMOTIC STUDIES

In order to obtain definite proof of the presence of a water-soluble lipase, enzymotic studies were made on the mycelium of *Penicillium roqueforti* grown for six days upon Czapek's solution. Ethyl butyrate to the extent of 0.5 c. c. per 100 c. c. of medium was added. Dox (4, p. 149) has shown that the addition of a particular substrate to a medium

will increase the production of the corresponding enzym. In accordance with this principle, the addition of ethyl butyrate should stimulate the production of lipase.

Preliminary experiments were conducted to discover the most active preparation of enzym. Three methods were tried:

(1) The "acetonedauerhefe" method of Albert, Büchner, and Rapp (1, p. 2376).

(2) Fresh mycelium was triturated with water and powdered glass, filtered, and the filtrate used.

(3) Washed, air-dried mycelium was triturated with dry powdered glass and the pulverized mixture used.

On tributyrin in water, blue with litmus, and with toluene as antiseptic, the "dauer" preparation gave no pink in six days, the water extract showed slight acidity in six days, and the fresh powdered mycelium showed a distinct acidity in 24 hours. Controls of boiled enzym preparations showed no acidity in six days. On ethyl butyrate made distinctly alkaline with one drop of decinormal sodium hydroxid the enzym extract showed acidity in 24 hours and the mycelium powder in 4 hours. Controls showed acidity in five days.

From these preliminary experiments the following conclusions were drawn:

The "dauer" preparation is unreliable for the study of lipase in the mycelium of *Penicillium roqueforti;* a water-soluble lipase can be extracted from the mycelium; and the most active preparation is the fresh pulverized mycelium.

A more accurate measure of the activity of the lipase at 38° C. to 40° C. was made on tributyrin, triacetin, ethyl butyrate, and ethyl acetate. For this study 1 gram of the air-dried mycelium was triturated with 4 grams of powdered glass, and 0.5 gram of the powder was added to 0.5 c. c. of the ester in 50 c. c. of distilled water contained in a small Erlenmeyer flask. The water was covered with a thin layer of toluene. Boiled enzym preparations were used in the controls. The flasks were tightly stoppered. At the end of two weeks the solutions were filtered and titrated with decinormal barium hydroxid. The results are given in Table VIII.

TABLE VIII.—*The action of the lipase of Penicillium roqueforti on esters.*

[Acidity in decinormal c. c.]

Ester.	Days.	Unboiled enzym.	Control, boiled enzym.
Tributyrin	14	0.67	0.08
Triacetin	14	.81	.51
Ethyl butyrate	14	1.37	.20
Ethyl acetate	14	1.31	.24

These enzymotic studies show that *Penicillium roqueforti* is well supplied with an enzym capable of hydrolyzing both simple esters and triglycerids.

CONCLUSIONS

The more important conclusions to be drawn from this investigation are:

(1) During the ripening of Roquefort cheese a considerable amount of the fat is hydrolyzed.

(2) *Penicillium roqueforti* produces a water-soluble lipase, which is the chief factor in the accomplishment of the hydrolysis.

(3) The hydrolysis results in the accumulation of the acids of milk fat in both the free and combined forms.

(4) Of these acids, caproic, caprylic, and capric and their readily hydrolyzable salts have a peppery taste and are responsible for the characteristic burning effect of Roquefort cheese upon the tongue and palate.

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CORN-LEAF BLOTCH MINER

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INTRODUCTION

There have heretofore been no references in the literature of economic entomology to *Agromyza parvicornis* Loew (Houser, 1912),[1] the corn-leaf blotch miner; therefore, it might be termed a new enemy of corn (*Zea mays*). While this is literally true, its work has been recorded previously, although credited to another species (Comstock, 1881). Up to the present time it has not proved to be a very serious pest, mainly because of the army of parasites that attack it. It is, however, entirely within the range of possibility that considerable injury may be done in the partial absence of these natural enemies. Every adverse influence tends to decrease the vitality of the plant, and, when a small plant of only three or four leaves has one or two of these destroyed, its metabolizing power is greatly lessened. Even large plants, if subjected to a heavy infestation of this insect, would undoubtedly suffer seriously, as each miner larva is capable of destroying half a square inch of leaf surface; and the injury is permanent, since the tissues die.

HISTORY OF THE SPECIES

The adult (Pl. III, fig. 1) was described in 1869 by Loew from a male and female from Washington, D. C. The late John B. Smith recorded it from New Jersey in 1909, but without reference to its habits. Prof. C. W. Johnson, of the Boston Museum of Natural History, stated in a letter to Prof. F. M. Webster, of the Bureau of Entomology, that he has collected this species at Niagara Falls, N. Y., and that Mr. S. A. Shaw took it at Hampton, N. H.

In 1879 Mr. Theo. Pergande, of the Bureau of Entomology, reared a dipterous miner from corn leaves collected in Washington, D. C. A record of this rearing was published (Comstock, 1881) under the name *Diastata*, n. sp. This material can not now be located, but the description of the mines would indicate that it was Agromyza and in all probability *A. parvicornis*.

During August, 1883, Mr. Pergande again reared the leaf-miner from corn leaves at Washington, D. C. Each time only a few Diptera were reared, though numerous parasites were obtained. D. W. Coquillett (1898) published the record of this second rearing under the name *Agromyza neptis*. He later wrote a marginal note on his copy of the

[1] Citations to literature in parentheses refer to "Literature cited," p. 30–31.

paper, changing the name to *A. parvicornis;* however, this correction appears never to have been published. The specimens have been examined by Mr. J. R. Malloch, recently of the Bureau of Entomology, who sustains the opinion of Coquillett. This is the only recorded injury that can be fixed with absolute certainty upon this latter species, although there is a record of the rearing of parasites from a leaf-miner on corn at Jacksonville, Fla., and a similar record by Prof. F. M. Webster at La Fayette, Ind.—both in 1886. The miner in both of these instances was probably *A. parvicornis*, as in these two records published in Insect Life[1] the host is given as *Diastata*, n. sp.

Wherever the work of a miner in corn leaves is mentioned the writer will state that it is probably *Agromyza parvicornis*, if the notes clearly state that it is a "blotch miner," since there is no other species of blotch miner known at this time to occur in corn leaves. This will explain why the work is attributed to this species, even when material has not been reared.

Dr. W. D. Hunter, in charge of Investigations of Insects Affecting Southern Field Crops in the Bureau of Entomology, collected this species at Victoria, Tex., in 1903, and Mr. E. S. Tucker, then of the same office, found it at Plano, Tex., in 1907.

The writer's attention was first called to this leaf-miner in June, 1908, at Richmond, Ind. While walking through a cornfield, it was noticed that the tips of some of the leaves appeared colorless, a few having a scorched appearance. Upon closer examination it was found that the tips of these leaves contained footless maggots which were devouring all the tissue between the two surfaces of the leaf, leaving it with a sickly, colorless appearance. Some of these larvæ were reared to adults and determined as *Agromyza parvicornis*.

Mr. C. N. Ainslie, of the Bureau of Entomology, noticed a leaf-miner in corn at East Grand Forks, Minn., in August, 1907, but reared only parasites. This may have been the same species as that found at Richmond, Ind., but, as no description of the mine was given, it is hazardous even to make a guess.

Mr. G. G. Ainslie, of the Bureau of Entomology, observed the work of a miner in corn leaves at Monetta, S. C., in May, 1908, and at Spartanburg and Clemson College, S. C., in June of the same year; but no adult flies were reared, although parasites were reared at Monetta and Clemson College. The species grown at Monetta, at least, was very probably *Agromyza parvicornis*, since its work was described as a "blotch mine." Mr. Ainslie reared adults of this species from corn at Marion, S. C., in May, 1909. Parasites were very numerous here, and only a few miners completed their development. A number of parasites were reared by Mr. Ainslie from a leaf-miner in corn leaves at Hurricane Mills, Tenn.,

[1] Some of the bred parasitic Hymenoptera in the national collection. U. S. Dept. Agr., Div. Ent., Insect Life, v. 2, no. 11/12, p. 348-353. 1890. "*Bracon diastatus* Ashm.," p. 348.

in 1911. From the appearance of the mines and pupa the host was probably *A. parvicornis*. He also reared adults of this miner from corn at Montgomery, Ala., in 1911 and at Lakeland, Fla., in 1912.

Mr. T. H. Parks, recently of the Bureau of Entomology, observed the work of a leaf-miner on sweet corn at Wellington, Kans., in 1909. While nothing but parasites was reared, the character of the mine indicates that it was probably the work of *Agromyza parvicornis*.

This species was again found at Richmond, Ind., but sparingly, in 1909, 1910, and 1911. In October, 1911, it was found infesting broom or hog millet (*Panicum miliaceum*) at La Fayette, Ind., and in 1912 it was very abundant in corn and several of the grasses on the experiment station grounds at La Fayette. In fact, it could be readily found in any cornfield in that locality.

Mr. J. J. Davis, of the Bureau of Entomology, found it at Lancaster, Wis., mining in corn leaves, and at Danville, Ill., in the leaves of *Echinochloa crus-galli*, in July, 1912. Mr. C. N. Ainslie reared the same miner from corn at Salt Lake City, Utah, in 1912; and Mr. W. L. McAtee, of the Biological Survey, collected it at Biltmore, N. C., the same year. It appears to have been more abundant in 1912 than during any of the other years, and naturally more species of parasites were reared at that time than from all previous rearings. Mr. Davis also found the work of what is probably this species near Louisville, Ky., in July, 1913.

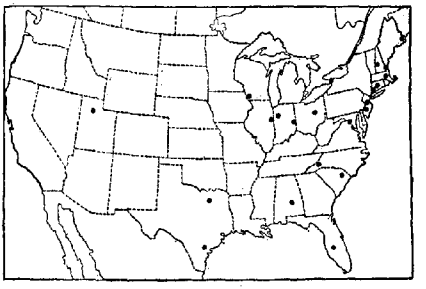

FIG. 1.—Map of the United States, showing distribution of *Agromyza parvicornis*, the corn-leaf blotch miner. (Original.)

DISTRIBUTION OF THE CORN-LEAF BLOTCH MINER

From the foregoing it will be seen that this leaf-miner has quite a wide range, being found as far north as Wisconsin, as far east as Washington, D. C., and New England, as far south as Alabama and Florida, and as far west as Salt Lake City, Utah, as well as in Texas. It probably occurs throughout the United States wherever corn is grown. The known distribution is shown on the map (fig. 1).

HOST PLANTS

There does not seem to be a great variety of host plants, *Agromyza parvicornis* having apparently thus far confined itself to a few species of the Gramineæ. The species seems to show a preference for corn, especially

the young plants, although it is very partial also to some of the broad-leaved millets. On corn it breeds continuously throughout the season. The writer found larvæ in mines in corn leaves from May until severe frosts killed the plants in the fall. Next to young corn the blotch miner apparently prefers the broad, hairy-leaved varieties of millet, such as *Panicum miliaceum*, although it has been reared from several varieties of the smooth-leaved millets. Occasionally it will be found in crab-grass (*Panicum sanguinale*), and it breeds readily in barnyard grass (*Echinochloa crus-galli*). It would not be at all surprising if in later years it should be reared from wheat and oats, as apparently the same kind of larval mine has been found in these plants, although the adults have not been obtained.[1]

CHARACTER OF INJURY

In young corn plants and in the small grasses and grains the larvæ work from the tip of the leaf toward the base, devouring all of the tissue between the upper and lower epidermis (Pl. I, figs. 2, 3). The mandibles of the larva consist of two chitinized hooks, which are used in much the same manner as a hoe, portions of the thin layer of tissue being scraped from between the two surfaces of the leaf with every stroke. In small plants the larvæ work the entire width of the leaf, leaving only the epidermis of the upper and lower surfaces. Plate I, figure 2, shows a young corn leaf taken from a cage, with six larvæ at work inside. Soon after the work is done the leaves have a colorless appearance, and in a few days they turn brown and curl up. One larva is sufficient to destroy a young corn leaf, although as many as four have been found in a single leaf in the field, and as many as eight or ten have been found in a leaf in the rearing cages. Often there is not sufficient nourishment in a single leaf for the development of all when they are reared in confinement, in which case some perish. Sometimes in the field two or more leaves of a young corn plant are attacked when the plant is only a few inches high and has, therefore, few leaves. It can be readily seen that corn might suffer severely in a cold, backward season with this pest abundant.

Instances have been noted where the tip of the mined leaf has been almost completely filled with water, although the larva may still be inside and apparently not seriously inconvenienced. Plate I, figure 3, shows such an instance, the epidermis of the upper and lower surfaces being separated for a distance of about one-fourth of an inch. The mines become filled in this way only when the weather is very rainy. In cases of this kind the larvæ do not feed much until the water has evaporated, although under normal conditions they appear to feed continuously, the writer having observed them both night and day and never having seen them at rest for more than a few seconds at a time.

[1] It might be well to state in this connection that Asa Fitch (1856) reared and described a new species of Agromyza (*A. tritici*) from New York, calling it "the wheat mow fly." After reading the description in Fitch's article and comparing it with the work of the miner at La Fayette, Ind., one readily sees that two species are involved.

The leaves of the grasses and smaller grains are affected in the same manner as the leaves of young corn, but as they are much smaller they can not accommodate so many larvæ. This miner is not able to go down the base of one leaf and up into another as is *Ceradontha dorsalis* Loew; therefore it has to depend entirely upon the nourishment to be derived from one leaf. *C. dorsalis* makes a long, narrow mine, working toward the base of the leaf, and in small plants it may go down the leaf sheath and work out into another leaf.

From the time corn leaves are an inch or more in diameter until they are mature the larvæ have plenty of room for development. The mines take on a different character in large leaves. In these they may start at any point along the leaf. Sometimes several larvæ will hatch in close proximity, and the mines will coalesce, forming a large blotch, which may be several inches in length and nearly an inch across. A mine like this is shown in Plate I, figures 5 and 6. At a distance of 10 or 15 yards such mines show up very distinctly against the dark-green background as large grayish or whitish blotches. Where there is only one larva to a mine in these large leaves the mine may be a comparatively long, narrow one (3 to 4 inches in length), gradually enlarging, the last third to half forming a blotch. In other instances the mine may be 3 inches in length and about three-eighths of an inch across for nearly the entire length, ending in a blotch about one-half inch in diameter, as shown in Plate I, figure 7. The mines may, however, form blotches, as shown in Plate I, figure 1.

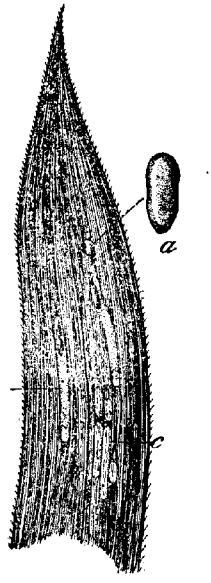

FIG. 2.—Tip of corn leaf, showing work of *Agromyza parvicornis*, the corn-leaf blotch miner: *a*, Egg, greatly enlarged; *b*, newly hatched larva in mine; *c*, feeding punctures of adults. Much enlarged. (Original.)

When the plants become older and tougher, the miners do not devour all of the tissue between the two leaf surfaces. In this case a miner that is working in the underside of the leaf would scarcely be noticed from the upper surface. Therefore, the greatest injury is wrought when the plants are young. If very abundant, however, the larvæ could cause serious injury to corn in advanced stages of growth, provided their parasitic enemies were not present in sufficient numbers to hold them in check.

DESCRIPTION OF AGROMYZA PARVICORNIS

THE EGG (FIG. 2, *a*)

The egg is milky white and flattened from above and below. It is from 0.4521 to 0.5043 mm. in length and from 0.1739 to 0.1913 mm. in width. It is broadly rounded at each extremity and slightly constricted at the center. The anterior extremity is slightly more pointed and somewhat more flattened. The surface of the chorion is smooth and apparently without any markings whatever.

Mr. J. R. Malloch, formerly of the Bureau of Entomology, has kindly consented to draw up descriptions of the larva and pupa. He has redescribed the adults also. His descriptions follow.

THE LARVA (FIGS. 3 AND 4)

In color the larva is pale greenish white, becoming, as it nears maturity, more yellowish white. The average length when mature is about 3 mm. and the breadth about 1 mm. The segments are but poorly defined and under a strong magnification show on the surfaces shallow, closely placed punctures, in each of which is situated a very minute hair. The mouth parts are heavily chitinized, deep black, and, while capable of being entirely retracted, are always visible, as they show clearly through the semitransparent larval skin. The spiracles of the anterior pair are small and slightly darker than the general color of the larva; the posterior pair, which are more or less embedded in the body, are closely approximated and placed at the extreme end of the body. On the ventral surface close to the anal extremity there is a vestigial sucker-like foot.

FIG. 3.—*Agromyza parvicornis*, the corn-leaf blotch miner: Full-grown larva; *a. sp.*, anterior spiracles; *p. sp.*, posterior spiracles. Much enlarged. (Original.)

THE PUPARIUM (FIG. 5)

In color the puparium is reddish brown. The length averages about 3 mm. and the breadth slightly over 1 mm. The segments are well differentiated, without any ornamentation or punctation. The spiracles of the anterior pair are prominent and protruding, those of the posterior pair (fig. 5, *c*) much more closely placed and less distinct. In shape the puparium is as represented in figure 5, *a* and *b*.

THE ADULT (PL. III, FIG. 1)

Agromyza parvicornis Loew, 1869, *in* Berlin. Ent. Ztschr., Jahrg. 13, No. 92, p. 49.

MALE AND FEMALE.—Frons black or black brown, opaque; orbits slightly shining black, four orbital bristles present; orbits differentiated from center stripe, bristles situated nearer inner than outer margin of orbits, a few weak hairs in an irregular row laterally beyond them. Antennæ brown or brownish black, rather below the normal size; third joint short, rounded in front, thickly covered with short, soft, whitish pilosity; arista brown, generally yellowish near base, except on the short thickened portion, which is glossy black; pubescence very close, generally distinct; length of arista equal to the distance from its base to the upper orbital bristle. Face brown, nearly perpendicular in profile, the central keel slight; cheeks brown or yellowish brown, very much higher posteriorly than anteriorly, at highest part one-third as high as eye; marginal bristles numerous; vibrissa differentiated but not very strong; proboscis brown; palpi black, very slightly dilated, weakly bristled. Mesonotum glossy black; disk thickly covered with short setulæ; two pairs of dorso-centrals present; the bristles between the posterior pair distinct; pleuræ, scutellum, and postnotum concolorous with disk of mesonotum, pleural sutures rarely, and beneath wing bases generally yellowish; squamæ whitish yellow, fringes brown. Abdomen colored as the thorax; ovipositor of female as in Plate III, figure 1, *E;* hypopygium of male as in Plate III, figure 1, *D*. Legs black, the tibiæ and tarsi sometimes paler, brownish

FIG. 4.—*Agromyza parvicornis*, the corn-leaf blotch miner: Mouth hooks of larva. Greatly enlarged. (Original.)

yellow, most distinct on knee joints; mid tibia with the posterior bristles distinct. Wings clear, slightly grayish on anterior half; venation as in Plate III, figure 1, *A*; halteres yellow, the knob whitish. Length, 3 to 4 mm.

Originally described from the District of Columbia (Osten-Sacken).

LIFE HISTORY OF AGROMYZA PARVICORNIS

OVIPOSITION

The act of oviposition has never been observed by the author, although the females have often been seen making feeding punctures which are apparently the same as egg punctures.

The eggs, which have been observed in the leaves repeatedly, may be deposited either from the upper or lower surface. The females always choose the tip of the leaf in small plants when ovipositing in the field, but in large plants the eggs may be placed at any point on the leaf. In confinement they seek the tips of the small leaves also, but as there is necessarily a small amount of leaf surface in a small cage, there are often as many as 15 eggs deposited in one leaf at different points. The eggs are inserted with the long axis parallel to the veins of the leaf. Figure 2, *a*, represents the eggs in situ and also greatly enlarged.

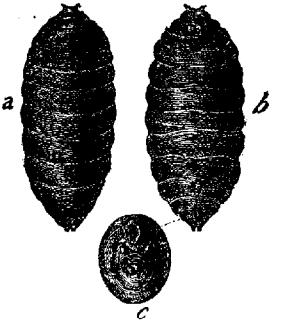

FIG. 5.—*Agromyza parvicornis*, the corn-leaf blotch miner: Puparium; *a*, dorsal view; *b*, ventral view; *c*, view of posterior extremity. Much enlarged. (Original.)

In making the puncture the fly forces the point of her abdomen downward, rearing the anterior portion of her body slightly, and touches the tip of the abdomen to the leaf, whereupon the small lancets, which apparently make up the ovipositor, are put in motion. The lancets appear to slide past each other, with their cutting edge in the plant tissues, thus acting somewhat like a saw. They are forced down between the two surfaces of the leaf and a strip of the epidermis about 0.3 mm. in width and about 0.9 mm. long is pushed back; the egg is probably inserted then, and this flap is in some way brought back over the egg and fastened down, probably with a mucilaginous substance. Sometimes it appears as though both ends of the egg were covered, although eggs are often seen to be only partly covered. These eggs can be readily detected with the naked eye when only a few feet away, as they show as milky white spots against the green background.

When only feeding, the female, after making the puncture, steps backward a few paces, inserts her proboscis into the puncture, and sucks the juices. The male has never been observed feeding at these punctures. The feeding punctures are very numerous, sometimes small leaves being

riddled even in the fields. Plate I, figure 4, illustrates this work in a leaf of millet as it occurs in the fields. In fact, in almost any place in the fields where eggs may be found there are usually one or more of these feeding punctures near by. Figure 2, *a*, *b*, *c*, represents the feeding punctures, eggs, and newly hatched larvæ as one often finds them in the field. These spots or punctures finally dry out and turn almost white.

Oviposition is, in all probability, accomplished during the day. Observations were made several times at night, but the adults were almost always at rest upon the plant at the top of the cage.

PERIOD OF INCUBATION OF THE EGG

No exact incubation period can be given, since this depends mainly upon the temperature. It is quite short in the summer, being about 80 hours in the latter part of July. In the latter part of September and the first part of October the eggs were not less than five days in hatching, and perhaps slightly longer. This would be nearly two days longer than the time required for hatching in July.

LENGTH OF LARVAL STAGE

The larval period, like the egg stage, varies considerably in length. In the middle of the summer larvæ will attain full growth in four days, while in the cooler weather of spring or fall they will require 10 days or more. Some larvæ hatched the first week in October and were overtaken by frosts.

LARVAL HABITS

When ready to hatch, the larva ruptures the eggshell at the cephalic extremity and begins to feed, gradually working its way out of the shell into its excavated burrow. It can not leave one leaf and enter another, and if removed from the leaf it will die. If a full-grown larva be taken from its burrow and placed upon a hard surface, it will bring each extremity beneath its body, then suddenly straighten to its full length, and thus skip about over the surface like a cheese maggot. Larvæ will continue this skipping for a considerable time, often jumping an inch high, apparently hunting for a hiding place. They may remain entirely submerged in water for a day or more and still recover and pupate normally. Excrement is voided at intervals within the mine, but at no time has a cast skin been found or other evidence of molting. The larva apparently punctures one of the walls of the mine and drops to the ground to pupate, going down sometimes 2 inches if the soil be sandy; in ordinary soil it will go only from one-fourth to one-half inch below ground. Larvæ will pupate on a solid surface in the open if they can not reach the ground or find cover. When ready to pupate the larva contracts in length, the segments becoming quite distinct. It then turns a cream color, gradually darkening until it becomes a reddish brown.

LENGTH OF PUPAL STAGE

The pupal stage varies from 14 days in warm weather to 22 days in spring and fall. In the late summer of 1912 there were some puparia that did not disclose adults, and upon examination they were found to be in good condition in November. These puparia were kept indoors and disclosed adults in January and February. They were of the generation that pupated from the middle to the latter part of August. This would seem to indicate that some go into hibernation rather early in the fall.

LIFE AND HABITS OF THE ADULTS

The flies begin to issue during the latter part of May. The males will not live more than three or four days in confinement, whereas the females will easily live two weeks when the weather is not too hot. It will thus be readily seen why the generations overlap so completely, since the progeny of one individual may be in the pupal stage before she ceases oviposition. The males apparently do not feed, though the females do considerable feeding, as evidenced by the punctures in the leaves shown in Plate I, figure 4. Both males and females, however, appear to be fond of water and seem particularly thirsty soon after issuing. In midsummer the adults appear to be most active in early forenoon and late afternoon. In midday they appear to seek shelter on the underside of leaves.

NUMBER OF EGGS DEPOSITED BY ONE INDIVIDUAL

The female flies begin oviposition in 5 to 10 days after emergence, if males be present; otherwise they will refuse to oviposit. There are no very complete observations on egg laying. Several individuals were observed, but for various reasons the records are not complete for their whole life. They have been found to deposit from 30 to 60 eggs, but will doubtless deposit more than this throughout the whole period. Dissections give no satisfactory clue to the number of eggs that may be deposited. The species will apparently breed as freely in confinement as out of doors.

NUMBER OF GENERATIONS

It is almost impossible to get a clue to the number of generations by making observations in the field, since they overlap so completely. After the first generation, if the insects are at all abundant, eggs, larvæ, and adults can be found in the field simultaneously throughout the remainder of the year. Therefore, early in the spring of 1912 a series of rearings was begun in confinement, and this series was continued until freezes killed off the vegetation in the fall. It was found that there were four complete generations and a partial fifth. It is possible that the insects may breed more rapidly in the open fields and that if they could be readily followed it would be found that there are five generations.

HIBERNATION

The first freezes in autumn killed the adults in the rearing cages at La Fayette, Ind., in 1912. Any larvæ that happened still to be feeding were killed also. This indicates very strongly that the species does not hibernate in either the adult or larval stage. It appears, then, that in the latitude of La Fayette, Ind., at least, it passes the winter in the puparium only.

LIFE HISTORY OF AGROMYZA PARVICORNIS IN FLORIDA

Mr. G. G. Ainslie made observations on this miner from November, 1912, to the middle of April, 1913, at Lakeland and Orlando, Fla. Special plantings of corn were made in the field, and volunteer plants and regular plantings were also kept under observation.

SEASONAL OCCURRENCE OF LARVÆ

On November 26, 1912, larvæ were found to be quite plentiful in volunteer corn that was about 10 to 12 inches high. This corn was kept under observation, and it was found that the miners bred slowly here throughout the winter. No killing frosts occurred during this time. There were three dates during this period at which the larvæ were the most abundant—viz, February 4, March 5, and March 27.

PERIOD OF INCUBATION OF EGGS

Of 19 eggs that were deposited in confinement during March, 2 hatched in 6 days and 17 in 7, while some that were found in the field did not hatch for 8 days, the average being 6.5 days.

LENGTH OF LARVAL STAGE

Accurate observations were made on 13 larvæ during the latter part of February and to the middle of March. It was found that the period from hatching until they left the mine varied from 6 to 12 days, the average for the 13 being 7.9 days. The larvæ in Florida, as well as farther north, invariably leave the mines to pupate.

LENGTH OF PUPAL STAGE

Table I gives most of the available data on the length of the pupal stage for Florida.

It will be noted that the larval stage was longer in Florida than given for Indiana. Larvæ that hatched late in Indiana were overtaken by frosts, while the rearings in Florida continued through cool weather without being interfered with by frosts.

The same interesting phenomenon occurred here that was observed in Indiana—namely, that apparently healthy living pupæ went into hibernation at a time when the miner was found breeding freely in the same

locality. Some individuals that pupated in January and February were apparently alive and in good condition as late as the 25th of April.

TABLE I.—*Length of pupal stage of Agromyza parvicornis in Florida.*

Number of individuals.	[1] Pupated—	Adult emerged—	Length of stage.
			Days.
1	Feb. 1	Mar. 4	32
1	1	4	32
1	20	16	24
1	20	19	27
1	Mar. 8	30	22
1	13	Apr. 3	20
6	.....	.....	157
Average length of pupal stage for 6 individuals, 26.2 days.			

[1] Two individuals pupated on Jan. 24 were still alive and healthy on Mar. 22.

In Indiana, as stated previously in this paper, individuals that pupated in the middle of August, 1912, and had been kept indoors since fall, disclosed adults in January and February, 1913.

The Florida data show conclusively that this insect will breed continuously, except in a few isolated instances, throughout the year, if its host plants are supplied and no freezing temperatures are encountered.

REARING METHODS

Several kinds of rearing cages were tried in this work, but it was found that the use of the one shown in Plate II, figure 2, was attended with the greatest success. It consists of a 12-inch flowerpot and a collar made of fine-mesh brass strainer wire with supports of galvanized iron, into which is fitted a large electric-light globe—the kind used on street lights—the top of which is covered with cheesecloth. There is a free circulation of air through the cage and moisture does not collect on the sides so readily as in other types, thus giving more nearly normal conditions. These cages were kept under a shelter so that direct rays of the sun did not strike them, and the miners were very easily reared in them. Plate II, figure 1, illustrates the rearing shelter with the cages in place.

PARASITIC ENEMIES

There are 18 species of hymenopterous parasites that may be said with reasonable certainty to attack *Agromyza parvicornis*, since there is as yet no proof that any are secondary parasites. Three of these are

braconids and 15 chalcidoids. Nine of these chalcidoids are known to be new species, and they have recently been described by Mr. J. C. Crawford, Associate Curator of the Division of Insects, United States National Museum. Three others are probably new, but as there is only a limited amount of material they can not be recorded as new with certainty. Two of the braconids are new also.

With this army of parasites in the field it will be readily seen that the chances that the miner will do serious mischief are reduced to a minimum. Only under exceptional circumstances would *Agromyza parvicornis* be able to elude this array of enemies. This is a very good illustration of the holding in check by its natural enemies of what would otherwise in all probability be a pernicious insect, thus showing that it is entirely possible for a group of parasites under favorable conditions to control their host insect.

The life history of none of these parasites has been worked out completely.

Of these parasites *Derostenus diastatae* is by far the most abundant and probably the most important. *Diaulinus websteri* and *D. begini* have also been reared quite plentifully and are probably next in importance. An effort is here made to discuss these parasites in the order of their importance without regard to their systematic relationship.

Derostenus diastatae How.—This chalcidoid very closely resembles *D. punctiventris* (Pl. V, fig. 1). It is an internal parasite and was first reared in 1879 from a corn-leaf miner which was called at the time *Diastata*, n. sp., but which has since been determined as *Agromyza parvicornis*. The parasite was described by Dr. L. O. Howard as *Entedon diastatae*. It appears to have been very abundant at that time. Prof. Comstock (1881) writes:

> During the season of 1880 these leaf-miners were extremely difficult to find, which was doubtless owing to the very extensive parasitization of the 1879 individuals. Out of thirty or forty specimens examined but one contained a sound larva, which was reared to maturity. All the rest contained several minute parasitic larvæ.

Prof. F. M. Webster reared this parasite in 1886 at La Fayette, Ind., from what was probably this miner. He reared large numbers of the same species at Urbana, Ill., in 1902, from a miner in grass. It was reared by Mr. C. N. Ainslie at Washington, D. C., in 1907, and by Dr. H. Kraemer, of Philadelphia, in 1905. In these two last instances it was parasitic upon a miner in grass leaves. Mr. G. G. Ainslie reared what is doubtfully the same species at Clemson College, S. C., in 1908. The host in this instance was probably *Agromyza parvicornis*, as it was a blotch miner in corn. He also reared it in abundance in 1911 and 1912 from a miner in corn leaves at Hurricane Mills, Tenn. Mr. T. H. Parks reared numbers of this parasite from a blotch miner in corn leaves at Wellington, Kans., in 1909. The same parasite was reared in abundance by the author from *A. parvicornis* at Richmond, Ind., in 1911, and at

La Fayette, Ind., both by the author and by Mr. Philip Luginbill, in 1912, while it was reared by Mr. G. G. Ainslie at Lakeland, Fla., and by Mr. J. J. Davis at Danville, Ill., in the same year. This parasite is thus known to cover a pretty wide range, and future rearings may show it to be present wherever its host is found. While nothing definite is known of its life history, it appears that the complete life cycle approximates that of its host. The larvæ of the parasite kill the larva of the host at or about the time the latter reaches maturity. Sometimes as many as eight of the larvæ of the parasite are found in one host larva. As soon as these are grown, they leave the body of the host and crawl out into the gallery a short distance to pupate. When they first pupate they are white, but later they turn black.

Diaulinus pulchripes Cwfd.—Mr. J. C. Crawford (1912) has recently described this species from two specimens in the Ashmead collection from Algonquin, Ill. Prof. Webster reared three specimens of this species from a miner in grass at Urbana, Ill., in August, 1902. The author reared a number of specimens from *Agromyza parvicornis* at La Fayette, Ind., in 1912. Nothing is known of its life history.

Diaulinus websteri Cwfd.—This species was reared quite plentifully from *Agromyza parvicornis* in corn leaves at Salt Lake City, Utah, in 1912, by Mr. C. N. Ainslie. It was recently described by Mr. Crawford (1912), the habitat being given as Tempe, Ariz.

Zagrammosoma multilineata Ashm.—Ashmead (1888) described this parasite under the genus Hippocephalus in 1888. The specimens were reared in 1887 from *Lithocolletis ornatella* Chambers on locust. Prof. Webster reared it from a species of Lithocolletis from Ohio in 1893. The author reared it from *Agromyza parvicornis* in 1912 at La Fayette, Ind. Nothing is known of its life history. Repeated attempts were made by the author to rear it, as it appeared to be quite common, but unless the pupæ were collected just before the adults were ready to issue they would not emerge.

Sympiesis sp.—This parasite was quite common at La Fayette, Ind. It seems, however, that for some unknown cause only a very few females were reared; consequently they have not been determined specifically. Doubtless they are new. Prof. Webster reared *Sympiesis nigripes* Ashm. from a lepidopterous leaf-miner in bur oak in Ohio in 1893. Mr. Parks reared a species of Sympiesis in 1909 from a blotch miner in corn at Wellington, Kans. Mr. G. G. Ainslie also reared it from the corn-leaf blotch miner at Hurricane Mills, Tenn., in 1912. The author reared it from *Agromyza parvicornis* at La Fayette, Ind. It is an internal parasite, but further than that nothing is known of its life history.

Closterocerus tricinctus Ashm.—This species (Pl. IV, fig. 1) was described by Ashmead (1888) under the genus Pleurotropis, with the statement that it was reared from a Lithocolletis larva on sycamore. Prof. Webster reared it from a miner in *Panicum multifolia* at Urbana,

Ill., in 1902, and Mr. G. G. Ainslie reared it from a miner in corn leaves at Hurricane Mills, Tenn., in 1911. The host in both instances may have been *Agromyza parvicornis*. The author reared it sparingly from *A. parvicornis* at La Fayette, Ind., in 1912.

Closterocerus utahensis Cwfd.—Mr. C. N. Ainslie reared this species from *Agromyza parvicornis* at Salt Lake City, Utah, in 1912. This is the only record of its attacking this miner. It had been reared from *A. pusilla* Meig. previous to this. Mr. Crawford (1912) described this species, giving the type locality as Salt Lake City, Utah.

Derostenus punctiventris Cwfd.—This is an internal parasite. It was reared by the author sparingly from *Agromyza parvicornis* at La Fayette, Ind., in 1912. Previously it had been reared from *A. pusilla* from several localities by other members of the force. It is a new species, and Mr. Crawford (1912) has recently described it, giving Salt Lake City, Utah, as the type locality. Plate V, figure 1, represents the adult of this species, which closely resembles *Derostenus diastatae*.

Diaulinus begini Ashm.—Mr. C. N. Ainslie reared this species sparingly from *Agromyza parvicornis* in corn leaves at Salt Lake City, Utah, in 1912. The author reared it from *A. pusilla* at La Fayette, Ind., in 1911. This species was described by Ashmead, and there was only one specimen in the National Museum collection previous to the recent rearings from Agromyza.

Notanisomorpha ainsliei Cwfd.—This parasite was reared sparingly from *Agromyza parvicornis* at Salt Lake City, Utah, in 1912, by Mr. C. N. Ainslie. It is a new species and was recently described by Mr. Crawford (1912). It has other hosts than *A. parvicornis*.

Cirrospilus flavoviridis Cwfd.—This is a new species and has recently been described by Mr. J. C. Crawford (1913). It has been reared from *Agromyza parvicornis* and *A. pusilla* at Salt Lake City, Utah, by Mr. C. N. Ainslie. Nothing is known of the life history.

Chrysocharis ainsliei Cwfd.—A few specimens of this parasite were reared from *Agromyza parvicornis* in corn leaves by Mr. C. N. Ainslie at Salt Lake City, Utah, in 1912. It has been reared from other species of Agromyza. It was recently described by Mr. Crawford (1912, p. 174), the type locality being given as Salt Lake City, Utah.

Chrysocharis parksi Cwfd.—One specimen of this parasite was reared from *Agromyza parvicornis* in corn leaves by Mr. C. N. Ainslie at Salt Lake City, Utah, in 1912. It was described in 1912 by Mr. Crawford (1912).

Pleurotropis utahensis Cwfd.—Mr. Crawford's (1913) description of this new species has recently been published. One specimen was reared by Mr. C. N. Ainslie from *A. parvicornis* in corn leaves at Salt Lake City, Utah. Mr. Ainslie also reared it from a species of Cephus in *Elymus* sp. in the same locality. Text figure 6 and Plate III, figure 2, represent the larva and adult, respectively.

Pteromalidæ.—There are at least two species of parasites belonging to the Pteromalidæ that have been reared from *Agromyza parvicornis* at Richmond and La Fayette, Ind. Both species may be and probably are new, but as there is only a limited amount of material they can not be placed with certainty. These parasites are mounted under Webster Nos. 3814 and 3857.

Opius diastatae Ashm.—This parasite (Pl. IV, fig. 2) was reared from a corn leaf-miner at La Fayette, Ind., in 1886, by Prof. Webster, and from a miner in corn leaves at Jacksonville, Fla., the same year by Ashmead. It was described by Ashmead (1888) as *Bracon diastatae*, and Gahan (1913) has placed it under the genus Opius. It is recorded in Insect Life[1] as being reared from *Diastata*, n. sp., which was very probably *Agromyza parvicornis*.

Opius succineus Gahan.—This is a new species recently described by Mr. A. B. Gahan (1913) of the Bureau of Entomology (Pl. IV, fig. 3). It was recorded from puparia by the author and Mr. Philip Luginbill from La Fayette, Ind., in 1912, and by Mr. J. J. Davis from Danville, Ill., during the same year. It was not very abundant, and nothing is known of its life history.

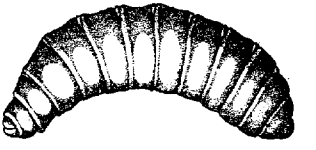

FIG. 6.—*Pleurotropis utahensis*, a parasite of *Agromyza parvicornis*: Larva. Much enlarged. (Original.)

Opius utahensis Gahan.—This species (Pl. V, fig. 2) is new also, having been described recently by Mr. Gahan (1913) from Salt Lake City. Mr. C. N. Ainslie reared this species sparingly from the locality just cited, from *Agromyza parvicornis*. Nothing is known of its life history.

Elachertinæ.—An unidentified species of the subfamily Elachertinæ was reared from a miner in *Panicum multifolia* at Urbana, Ill., in 1902, by Prof. Webster. As large numbers of *Derostenus diastatae* were reared from the same host at the same time and as this latter is the most important parasite of *Agromyza parvicornis*, it was thought well to mention in this connection the rearing of this unidentified species, which bears Webster No. 1896.

Macroglenes sp.—Mr. C. N. Ainslie reared a species of Macroglenes from a leaf-miner in corn at East Grand Forks, Minn. There is no way of knowing what miner was concerned, and this species as well as the foregoing is listed here mainly to establish a record of the rearings. The specimens bear Webster No. 4309.

REMEDIAL MEASURES

As no occasion demanding remedial measures has thus far arisen, little can be said on this subject. The ordinary means of control would appear

[1] Some of the bred parasitic Hymenoptera in the national collection. U. S. Dept. Agr., Div. Ent., Insect Life, v. 2, no. 11/12, p. 348-353, 1890. "*Bracon diastatae* Ashm.," p. 348.

to offer very little assistance, and it is difficult under such circumstances to suggest measures that would be effective when there has been no opportunity to put any of them into practice or to test their efficiency.

With such a host of parasites as are listed in the preceding pages constantly on the watch we need not concern ourselves seriously about remedies so long as conditions continue as they now are. In the event that a combination of circumstances should occur that would restrain the parasites and give free rein to their host, the blotch miner would undoubtedly prove a pest very difficult of control.

This species seems to furnish an instance in which only the barrier of parasites stands between the farmer and what may easily become, temporarily at least, a very serious pest.

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"*Derostenus punctiventris*, new species," p. 180.
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PLATE I

Fig. 1.—Large leaf showing blotch mines. Note feeding punctures and two eggs that have recently hatched, with short mines leading out from them.

Fig. 2.—Young corn leaf from breeding cage containing six larvæ.

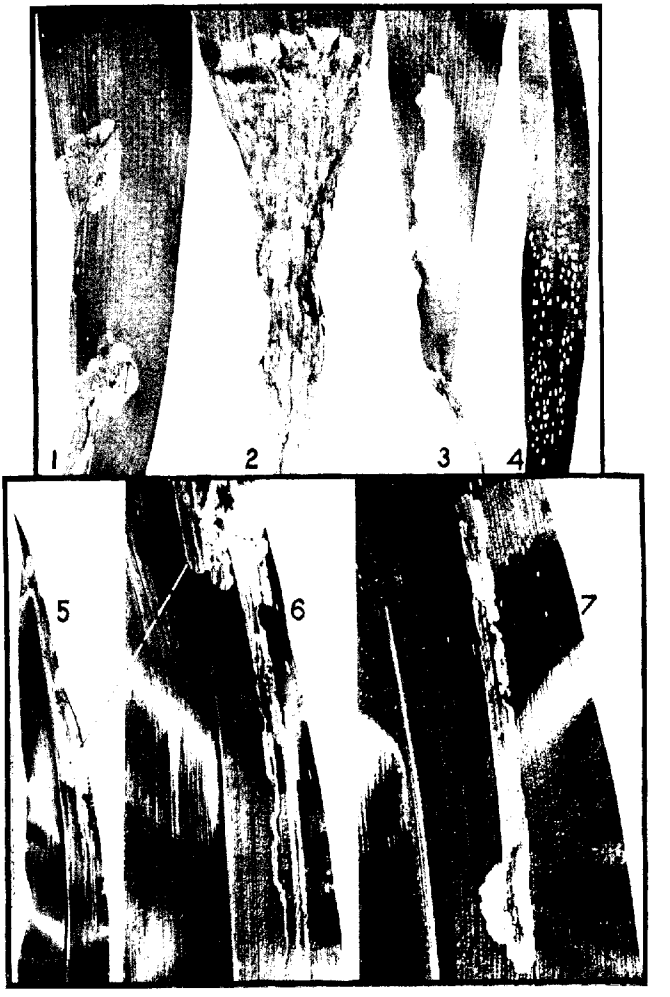
Fig. 3.—Shows how mines occasionally fill with water.

Fig. 4.—Millet leaf showing feeding punctures. All about natural size.

Fig. 5.—Large corn leaf with three larvæ in the same tunnel; reduced. Note point of origin of mines.

Fig. 6.—Section of leaf shown in figure 5 slightly enlarged.

Fig. 7.—Straight mine ending in a slight blotch in a large leaf; slightly enlarged.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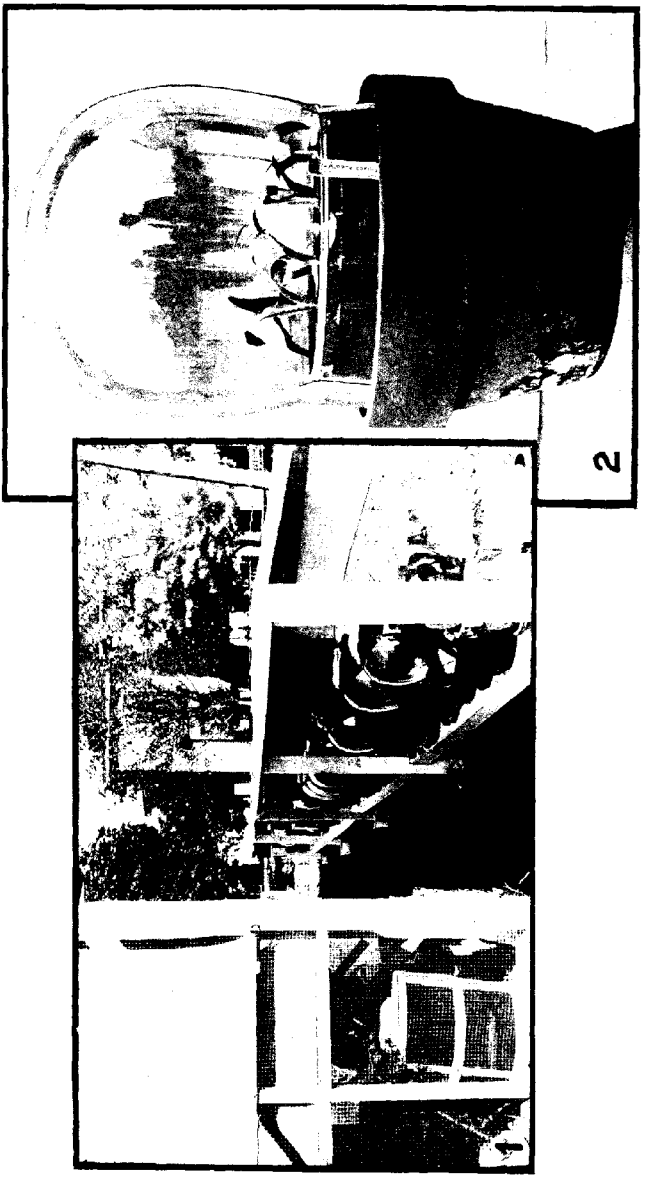
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PLATE II

Fig. 1.—Rearing shelter used for *Agromyza parvicornis* and other insects at La Fayette, Ind.

Fig. 2.—Rearing cage used for *Agromyza parvicornis*.

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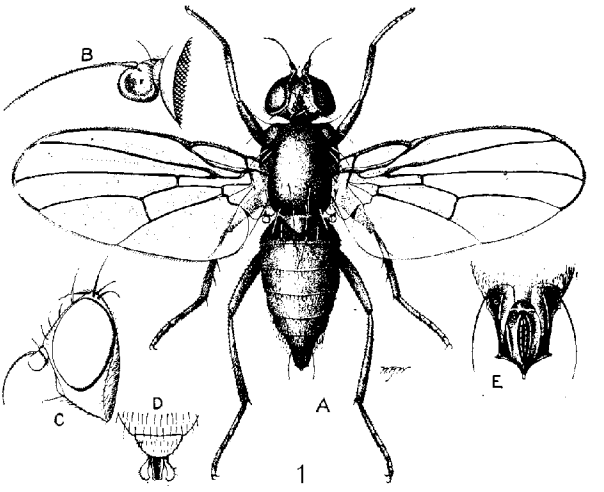
PLATE III

Fig. 1.—*Agromyza parvicornis*, the corn-leaf blotch miner: *A*, Dorsal view of adult; *B*, antenna of female; *C*, head of male; *D*, hypopygium of male; *E*, ovipositor of female. Much enlarged. (Original.)

Fig. 2.—*Pleurotropis utahensis*, a parasite of *Agromyza parvicornis:* Adult. Much enlarged. (Original.)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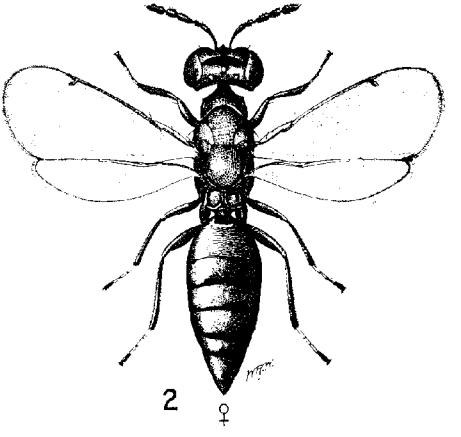
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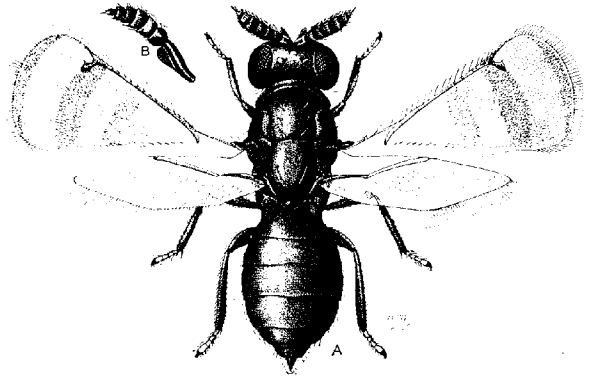

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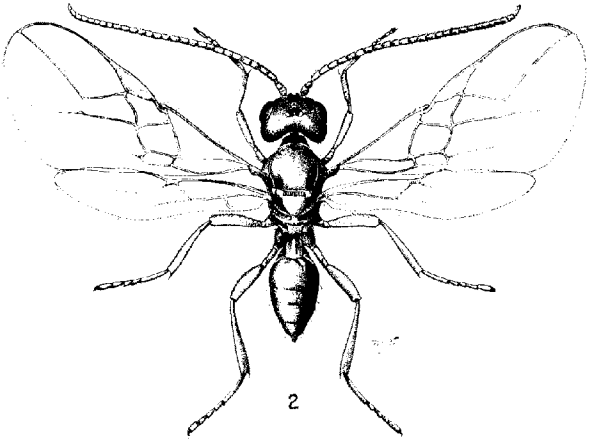

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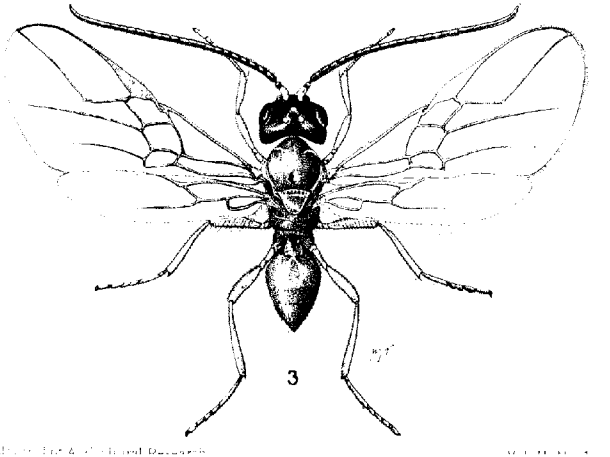

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PLATE IV

Fig. 1.—*Closterocerus tricinctus*, a parasite of *Agromyza parvicornis: A*, Dorsal view of adult; *B*, side view of antenna. Much enlarged. (Original.)

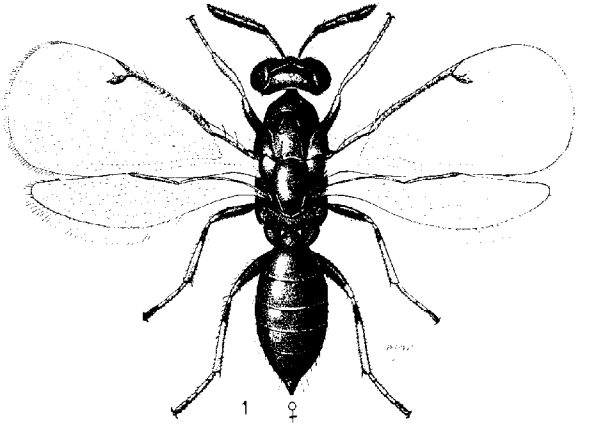
Fig. 2.—*Opius diastatae*, a parasite of *Agromyza parvicornis:* Adult. Much enlarged. (Original.)

Fig. 3.—*Opius succineus*, a parasite of *Agromyza parvicornis:* Adult. Much enlarged. Original.)

PLATE V

Fig. 1.—*Derostenus punctiventris*, a parasite of *Agromyza parvicornis*: Adult. Much enlarged. (Original.)

Fig. 2.—*Opius utahensis*, a parasite of *Agromyza parvicornis*: Adult. Much enlarged. (Original.)



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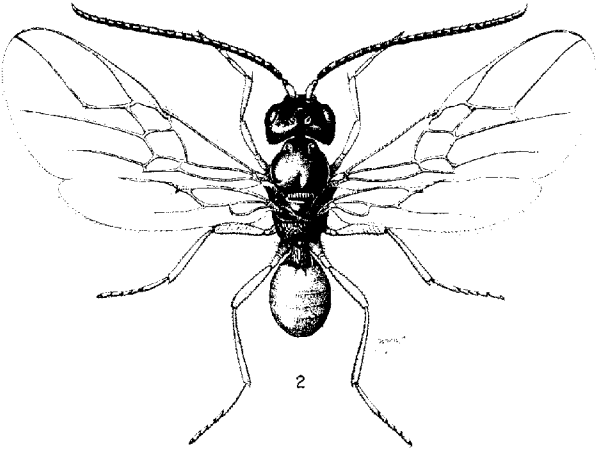

2

COLORATION OF THE SEED COAT OF COWPEAS

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INTRODUCTION

The following study of the seed coat of various cultivated cowpeas (*Vigna sinensis*) was made because the great diversity in color schemes and kinds of pigment in these seeds seems to have a direct bearing on problems of heredity, the pigmentation being to a large extent a basis for distinguishing one variety from another. It therefore seemed desirable that a clear understanding of the morphology of the seed coat and the way in which these pigments are arranged in its various layers should be obtained, in order to discover whether there are any facts bearing on problems of heredity, outside of the mere facts of the different color arrangements themselves. It also seemed not unlikely that such a study might prove that colors optically alike are in some cases different as to the material of the pigment and the place of its deposit.

METHODS OF PREPARATION

The best methods for the study of the cowpea pigmentation were found to be as follows:

The seed coats were removed from dry cowpeas in flakes as large as possible and were then cut transversely in pith, the sections made by hand being as thin as possible. It was found that to embed the seed coats for microtome sectioning necessitated subjecting them to water if the freezing process was used or to various solvents if celloidin or paraffin was used. Both methods resulted in dissolving the pigments to some extent and thereby causing them to appear in parts of the seed coat where they did not normally belong. The dry method of cutting avoided this difficulty. The sections were mounted dry under ⅞-inch square cover glasses, held in place by a drop of paraffin on either side of the glass. By this method the sections may be examined in the dry state and closely watched when water, various reagents, or stains are being applied, so that facts as to solubility, chemical reaction, etc., may be accurately noted. Such sections, held down by an immovable cover glass, are also ideal for high-power examination. If necessary, they may be also readily preserved for future study. Sections tangential to the surface of the seed coat were also made, but, aside from throwing light

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upon the structure of the cells in the various tissues, it was found that they were not so useful for an examination of the pigments and their distribution as sections made transverse to the seed coat or, in other words, perpendicular to the surface of the cowpea.

A large number of reagents were experimented with, but eventually it was found that those of practical use were extremely few—namely, distilled water, alcohol, ether, chloroform, xylol, solutions of caustic soda and caustic potash (the 1 per cent solutions being of greatest service), dilute hydrochloric acid, normal Fehling's solution, saturated aqueous solution of chloral hydrate, peroxid of hydrogen solution, and several stains, the most satisfactory being a 50 per cent alcohol solution of diamond fuchsin and a 10 per cent aqueous solution of pyronin. As above stated, these were used upon sections mounted dry under the cover glass, the various liquids being drawn through by means of triangular pieces of blotting paper placed at the opposite side of the cover glass. The length of treatment varied under different circumstances from a few seconds to 24 hours. However, most of the reactions that were significant were obtained within a few minutes, so that study could be rapidly carried on.

As the problems in mind had to do with the differences in the color schemes of cowpeas as a whole, only a general examination was made to learn in what respect different areas of the seed coat were differently pigmented. It became evident that although the pigment intensification varied in different areas of the seed coat, a general idea of the color scheme could best be found by studying sections taken from the side of the seed. The greater intensity of color around the hilum was found to be merely due to a larger quantity of the same pigments as those present on the side of the seed, and very frequently this heavier pigmentation proved to be a disadvantage, as, in the case of dark colors, they frequently obscured less intense pigments easily detected in sections made where coloration was not so dense. The only case where the pigmentation near the hilum was particularly worth studying was in those varieties where colors on the general surface were lacking—namely, in the cream-white and pure-white varieties. In this class the varieties that have a more or less intense pigmentation around the hilum give some information as to the tendency of general pigmentation that such a variety might be expected to show were the whole of the seed coat colored in the usual way.

MORPHOLOGY OF THE SEED COAT

Before discussing the coloration schemes in different varieties of the cowpea it is necessary to describe the structure of the seed coat, especially as seen in transverse sections. Such sections show that it may be divided into three layers. The outer layer is a single row of elongated palisade cells, with their long axis perpendicular to the surface of the seed

(fig. 1, *a*). Beneath this is a second layer, a single cell deep. Its cells are relatively cubical and have thick walls, but one horizontal diameter is slightly longer than the other. They are described by some authors as of hour-glass shape, a rather fanciful resemblance. They may be

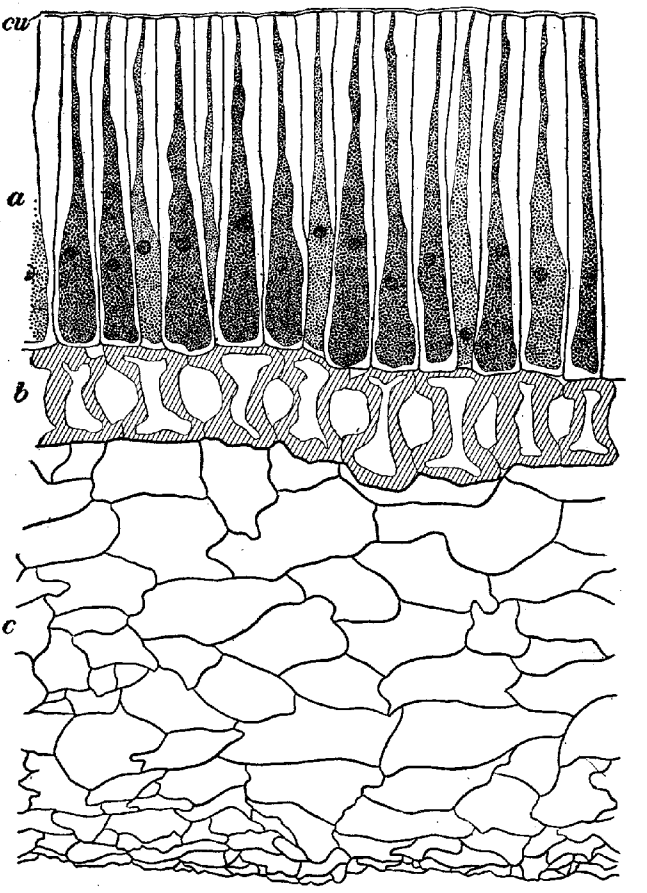


FIG. 1.—Transverse section of the seed coat of a cowpea with the cells expanded with chloral hydrate so as to show the structure of the three layers: *cu*, Cuticle; *a*, palisade layer; *b*, middle or hour-glass layer; *c*, basal-color layer.

said to lie at right angles to those of the palisade layer (fig. 1, *b*). Beneath this second layer is a comparatively thick layer from 10 to 20 cells deep, the cells being larger than those of the second layer and with relatively thin walls. These also lie parallel to the surface—that is, are hori-

zontal. They are considerably longer than broad and are, as a rule, so arranged that their longer horizontal axis is at right angles to the slightly longer horizontal axis of the cells of the second layer (fig. 1, *c*). One of the advantages of this arrangement may be to give tensile strength to the seed coat.

The middle layer of relatively cubical cells plays no rôle whatever in the pigmentation of the cowpea. Its cells are practically empty, only such residue of organic matter being present as would necessarily be found there. It is possible that there is some effect upon the coloration due to the included air which fills these cells and which in fresh-mounted sections always appears like a black band through the section; but, as the resulting color of the seed coat is made up from the different factors taken vertically, this single-celled empty layer between the palisade and the basal layers must have extremely little influence upon the color.

PIGMENTATION OF THE BASAL-COLOR LAYER

The third or inner layer is more or less filled with a pigment, which is the same in all the cowpeas examined, and for that reason the writer calls this the basal-color layer. The pigment is a melanin-like substance,[1] ranging from a pale-straw color to a deep orange or heavy buff. As a rule, it is massed in granular particles in the lower part of the layer. In some cases the upper cells contain the larger amount of pigment, and in a few instances it is evenly distributed throughout all the cells of the basal layer. In some cowpeas, and especially in those that have a heavy basal color, the pigment completely fills a large part of the cells and is then seen to be crystalline. In such cases the color is a deep orange or sometimes even a copper red. No trace in any instance was found of any other pigment in this basal layer. Anthocyanin tests failed in every case to give a reaction.

No attempt is made in this paper to discuss the cell contents of the seed coat, outside of those substances which are directly concerned in producing the color schemes found to exist in ripe cowpeas. How the various pigments arise in the growing cells and what are the mechanical principles back of their predetermined distribution in the different varieties are questions of great cytological interest, but not important for the subject in hand. It may, however, be worth while to mention here one substance which is very generally associated with the different pigments—namely, tannin. Tests with such reagents as ferric chlorid, ferric acetate, potassium bromate, osmic acid followed by hydrogen

[1] The applying of the term "melanin" to any plant pigment has been criticized. (*See* Gortner, R. A. The misuse of the term "melanin." Science, n. s., v. 36, no. 915, p. 52–53. 1912.) Although Mr. Gortner, so far as the writer knows, has not sufficient ground to warrant his exclusion of this term from plant nomenclature, seeing that the statement that it never occurs in plants is unproved, the writer agrees that its use here is open to criticism, and has therefore substituted "a melanin-like pigment" because no advantage can be found in employing Osborn's term "humin," favored by Gortner, the boundaries of this term being at present as vague and unsettled as those of melanin.

peroxid, or any of the alkaline carbonates demonstrate that one or more of the half-dozen tannins known to exist in plants are present in appreciable quantity in all the pigmented cells of the cowpea. But the optical color effect of this tannin is too small to need any attention here. Whatever rôle tannins may play in producing the pigments deposited in the cells,[1] their chief service in the mature seed coat is undoubtedly protective.

PIGMENTATION OF THE PALISADE LAYER

The upper or palisade layer plays a most important part in the pigmentation of the seed coat. As a rule, the cells are from 6 to 10 times as long as broad, with the cell cavity greatly enlarged at the lower or

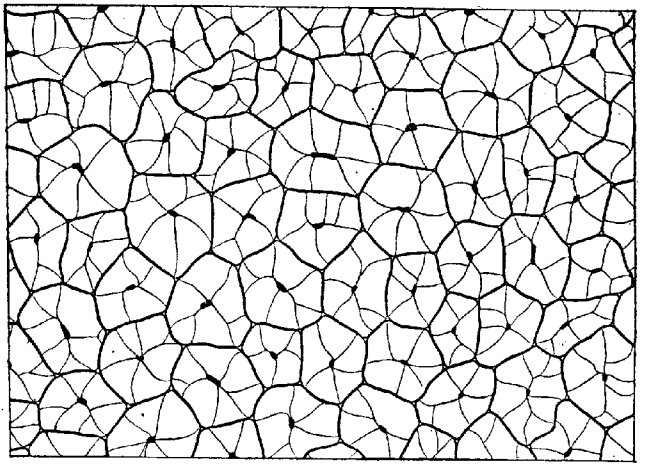


FIG. 2.—Outer surface of the seed coat of a cowpea, showing the upper ends of the palisade cells.

inner end, and gradually tapering upward to a mere thread at the upper or outer end of the cell (Pl. VI, fig. 1, *a*, and text fig. 1, *a*). From the central cavity, however, there radiate out into the gradually thickening walls from three to six vertical clefts, each reaching or nearly reaching the cell wall, thereby affording intercommunication between the cells. The result of this is that, looking on the cowpea seed coat from above, these cells appear to have at their outer end a stellate cavity (fig. 2). In focusing downward on the palisade cells these clefts gradually decrease by the widening of the central cell cavity until at or near the base the

[1] See an article on anthocyanin-forming bodies by Ioannes Politis, entitled "Sopra speciali corpi cellulari che formano Antocianine," published in Atti, R. Accad. Lincei, s. 5, Rend., Cl. Sci. Fis., Mat., e Nat., v. 20, sem. 1, p. 828–834, 1911. Politis here claims to have proved that anthocyanin is produced by certain special organs, cyanoplastids, in the composition of which tannin is a chief ingredient.

cavity occupies almost the entire width of the cell. The walls are of exceedingly dense cellulose. An outside cuticular sheath in rare cases is found covering the upper or outer surface of the palisade layer, but in most instances it is lacking (Pl. VI, fig. 1, *cu*), and the narrowed cavity of the cell seems to either reach or almost reach the outer surface of the seed coat. That there often is an actual aperture at the upper end of the cell is easily demonstrated; for when stains or colored reagents are used, it is easy to trace the rapid inflow of the liquid through these narrow, threadlike extensions of the cell cavity downward into the larger area at the lower end. Air bubbles are also seen to be pushed forward by the inflowing liquid and to pass downward into the large basal cavity. The bearing of this fact on the absorption of moisture necessary for the germinating of the seed is evident.

The normal form of the palisade cells in some varieties is strangely modified, the cell walls being very irregular. The taper of the cell cavity in such cases is imperfect, and after suddenly narrowing from the wide basal portion to a mere thread it again expands toward the outer end into a sort of mushroom-shaped enlargement. The cells themselves are also greatly contorted in outline and are sometimes spirally twisted upon their long axis, so that a true longitudinal section of these cells, such as is usually obtained in a transverse section of the seed coat, is quite impossible. Sometimes they are more or less intertwined. These distortions, as will be pointed out, are associated with certain color schemes and are quite characteristic of certain varieties.

There are two classes of pigment found in the palisade cells: First, a melanin-like pigment, identical in all its reactions and similar in its color to the pigment referred to in the lower of the three layers—namely, the basal-color layer. In some instances this is present in all the palisade cells, thereby supplementing and intensifying the basal color of the seed coat. In most cases it is confined to small groups of cells interspersed among larger or smaller areas of the palisade layer destitute of this pigment. According as this pigmentation is uniform or irregular in its deposit the basal color of the seed coat is uniform or mottled.

More frequent than this melanin-like pigment in the palisade layer are various anthocyanin pigments. These also may fill uniformly all the palisade cells or may be variously grouped and interspersed with colorless cells, thereby giving rise to the very diverse color schemes characteristic of different varieties of cowpeas. The anthocyanin pigments are practically of two kinds: First, an acid-reacting anthocyanin, ranging in color from a decided rose red to a strong purple; and, second, an alkaline-reacting anthocyanin, uniformly of a deep indigo blue, but which in mass often appears as dead black.

In many instances only one of these phases of anthocyanin pigment is discoverable in the seed coat of a given variety; and according as it fills

uniformly the cells of the palisade layer or is irregularly deposited, we have modifications in the color of the seed coat, giving rise to various forms of speckling, blotching, marbling, or monochrome coloration. In many cowpeas both the alkaline-reacting and the acid-reacting anthocyanin are present. As a rule, they are deposited in separate cells, but in a great many cases they are to be found in the same cell. When this latter is the case, the alkaline-reacting anthocyanin always occupies the lower half or third of the cell—that is, the part where the cavity is largest—and is collected in dense granular masses of a deep indigo blue. The rose-colored anthocyanin usually occupies the upper portion of the cell or occasionally fills more or less the entire cavity. The finding of both alkaline and acid reacting anthocyanin in the same cell is in harmony with a well-established cytological condition, namely, that one end of a cell may give an acid reaction while the other gives an alkaline one.

That these two phases of anthocyanin pigment are probably the same material is easily demonstrated. When thin transverse sections are suffused with neutral distilled water, the rose or purplish anthocyanin generally found in the upper end of the cell quickly diffuses into the surrounding liquid, thereby rendering more visible any alkaline indigo-blue anthocyanin which may occupy the lower portion of the cell. This latter, although also soluble in water, is very much slower in dissolving, taking several hours to disappear. Moreover, if such sections, instead of being treated with distilled water, are treated with a weak alkaline solution, such as a 1 per cent solution of caustic potash, both phases of anthocyanin undergo the same reactions. The rose-colored anthocyanin is immediately changed into an intense blue, and this, together with the indigo-blue anthocyanin, slowly passes through different shades of blue, green, greenish yellow, pale yellow, and finally is bleached and disappears. If, on the other hand, a weak solution of an acid is used, such as a 1 per cent solution of hydrochloric acid, both phases of anthocyanin again undergo the same reaction. The indigo blue immediately changes to an intense rose red and rapidly diffuses in the surrounding liquid. The tints assumed by these two phases of anthocyanin pigment are so perfectly identical with all the reagents that have been tried that it is fair to assume that we have essentially the same material in both cases, but in the one instance in an acid state and in the other in an alkaline state.

It will be seen that in all pigmentations of the palisade layer the colors are superimposed upon the underlying basal color of melanin-like pigment. It is by means of this palisade layer, therefore, that we secure the great diversity in color schemes characteristic of the cowpea. If the general pale-buff or orange-brown basal color is modified by even deposits of melanin-like pigment in the palisade layer, an intensification of the basal color is obtained, which sometimes amounts to a copper

red or dull reddish brown, uniformly spread over the seed coat. If the palisade layer contains uniformly anthocyanin in its cells, the basal color is obscured or modified by this superimposed pigment and assumes a blue or black or purple tint. If the palisade cells are irregularly pigmented, all of the modifications in marbling, speckling, and streaking which serve to distinguish the different varieties of cultivated cowpeas are found (Pl. VI, fig. 2). It may therefore be said in general that the diversification in color is principally brought about by the deposit in the palisade cells alone of the pigments above mentioned and that the different tints of color tones are the result of the various combinations of pigment already mentioned.

SEEDS DESTITUTE OF PIGMENTATION

Some reference should be made to such cowpeas as are more or less destitute of pigmentation. The writer examined only one cowpea the entire seed coat of which gives no evidence of pigment deposit; but among the large number of white and cream-white cowpeas there are probably several others quite destitute of pigmentation—that is, true albinos. In fact, four other varieties of white or cream-white cowpeas were examined in which the maximum quantity of pigment was so minute as to make its detection quite difficult, and certain individuals of these varieties, after the most painstaking tests, left the question in doubt as to whether or not even a trace of pigment was present. They were Nos. 212–2–11, 212–6–8, 213–2–4, 214–3–2Re. From the standpoint of coloration, therefore, these pure white and cream-white varieties may all be safely considered as albinos. The strict albino examined was No. 0632. Most careful testing failed to disclose any coloration in the palisade layer or in the basal-color layer, long treatment with various reagents resulting merely in such faint tints as would be secured by reactions on the normal cell contents, such as cytoplasm and nuclear substances. With the exception of these albinos, all the cowpeas examined have more or less pigment deposited in the basal-color layer, and, as before stated, this is of a melanin-like character. When the palisade layer is destitute of pigment, uniform tinted cowpeas are obtained, ranging from a cream-white seed coat, where the amount of pigment in the basal-color layer is very small, to a strong buff or even red brown, where the amount is greater.

After this general consideration of the structure of the seed coat and of the various ways in which its color layers are pigmented, the chief varieties of cultivated cowpeas may be described individually. The varieties here enumerated represent in a general way all the known color schemes found in cowpeas. They were chosen at the suggestion of Prof. W. J. Spillman, of the Bureau of Plant Industry, who proposed this line of investigation, these types being those used by him in connection with certain studies in hybridization.

CLASSIFICATION OF COLOR FACTORS IN COWPEAS

For convenience of reference, the foregoing factors of coloration in cowpeas may be classified as follows:

I. Basal-color layer.
  a. Devoid of pigment (white).
  b. With melanin-like pigment (buff to brown).
II. Palisade layer.
  A. Solid colors.
    a. Melanin-like pigments.
      a'. Buff, clay, etc.
      b'. Coffee, brown, etc.
    b. Anthocyanin pigments.
      a'. Red (acid state).
      b'. Blue (alkaline state).
      c'. Purple (combination of *a'* and *b'*).
      d'. Black (intensification of *b'* or *c'*).
  B. Variegated colors.
    a. Marbling.
      a'. Affects melanin-like pigments only. Whippoorwill type.
    b. Speckling.
      a'. Deep-blue anthocyanin in groups of cells, groups widely scattered. Taylor type.
      b'. Same, but groups more plentiful. New Era type.
    c. Marbling and speckling.
  C. Eyed.
    a. Watson type of eye. Margin of eye indefinite.
    b. Holstein type of eye. Seed of eye indefinite.
    c. Narrow eye. Narrow patch of color about the hilum indefinite at lower end (micropylar).
    d. Small eye, due to presence of both *a* and *b*.
    e. Very small eye, due to the presence of both *a* and *c*.
  D. Dilute colors.
    Characterized by individual unpigmented cells scattered among pigmented cells over entire seed coat.

CLASSIFICATION BASED ON DISTRIBUTION AND KINDS OF PIGMENTS

For convenience in grouping, the writer has divided the different varieties into four classes: (1) Those with or without a pigment in the basal-color layer, but none in the palisade layer; (2) those in which there is a pigment in the basal-color layer and anthocyanin only in the palisade layer; (3) those in which there is pigment in the basal-color layer and a melanin-like pigment only in the palisade layer; and (4) those in which there is a pigment in the basal-color layer and both anthocyanin and a melanin-like pigment in the palisade layer.

I.—COWPEAS HAVING NO PIGMENT IN THE PALISADE LAYER

Under the first division I have found, as previously stated, one cowpea, No. 0632, which is an extreme type of albinism. Here both the palisade and basal-color layers are destitute of all pigmentation. The

writer has not been able to discover the parentage of this variety. The reasons for its white color are two: First, the palisade cells are practically destitute of contents. Such residuary amount of cytoplasm as is present occupies a very minute part in the cell cavity and generally its upper third, instead of being in the lower end, as is usually the case. Parallel with this fact the usual spindle-shaped tapering of the cell cavity is here so slight that the diameter of the lower part is hardly greater than that of the upper part. In other words, the fine hairlike extension of the cell cavity upward does not exist. Near the upper part of the cell the somewhat narrow canal cavity widens out, and it is at this point that the small residue of cytoplasm is to be found. The cells are also more loosely bound together than usual, so that intercellular spaces between them are quite frequent. The second reason for the white color is that in the basal-color layer, which in most cowpeas is colored with a dense yellow or orange-buff pigment, there is no trace of pigment present nor any pigment reaction obtainable. The seed coat is, as a whole, much thinner and weaker than in other varieties, and its permeability to external moisture should therefore be greater.

As already stated, several other cowpeas approximate this true albino in being practically colorless, but certain individuals of these varieties show a slight trace of pigmentation in the basal-color layer. These varieties, 212–2–11, 212–6–8, 213–2–4, and 214–3–2Re, have the striking irregularity in form of the palisade cells and the lack of taper in the cell cavity just described in the case of No. 0632. Special mention should also be made of a somewhat analogous case in No. 239–5–3–18. This is also a cream-colored cowpea, but has a deep purplish pigmentation around the hilum, forming an "eye." In view of this localized pigmentation it is necessary to classify this variety under Division IV, the palisade cells in the area of the "eye" having both anthocyanin and melanin-like pigment. The color scheme of this portion of the seed coat will therefore be treated under Division IV, but as the structure of the uncolored seed coat, exclusive of the "eye," shows certain curious features identical with the white forms just described the case is here given for comparison. A transverse section of the cream-white seed coat of this cowpea shows remarkable contortion in all its layers. The palisade cells have very strongly marked the abnormal shape previously mentioned, having heavy walls and being shorter than usual; their form is irregular and twisted upon its axis. The cell cavity is very broad at the base, narrows suddenly at its middle, and again broadens slightly at the upper end. The very small residue of cytoplasm is generally found located in this upper widened portion, thus corresponding to the albino, No. 0632. Here also there is no appreciable trace of pigment to be found in the palisade cells. The cells of the remaining layers of the seed coat are also much contorted and have, in general, heavier walls than normally. A minute amount of pigment is present in the

basal-color layer and is contained in widely separated cells. It gives the same reaction as the yellow melanin-like pigment usually found in this layer. It is here, however, of a very light-straw color, this being due to the minute quantity rather than to any difference in character. This hybrid is the third generation of a cross between a Watson No. 5 and a Taylor No. 14. The significance of the contorted cells here mentioned should be borne in mind in view of its parentage, as it will be a subject for discussion under a later variety.

No. 237–3–7 is in its general color cream white, often intensified into buff, or even in a few individuals distinctly brown. The color is more conspicuous about the hilum. Therefore, it should be classified and described under Division III, although in general appearance it often seems to be uncolored.

II.—COWPEAS HAVING ONLY ANTHOCYANIN IN THE PALISADE LAYER

The second group of cowpeas is that having only anthocyanin in the palisade cells, with a melanin-like pigment always present in the basal-color layer. Nine varieties were found to have enough difference in color scheme to be separately examined. In the first, No. 243–1–5, the seed coat is a strong red, varying to purplish brown. The palisade cells are strongly pigmented with the general color of the seed coat, so that the basal-color layer, which has the usual orange-yellow pigment, probably has little part in the general coloration, being obscured by the heavy pigmentation of the palisade layer overlying it. In neutral water the palisade pigment appears as a dull rose and slowly dissolves. Various reagents show it to be anthocyanin. Possibly it is mixed with a minute trace of buff-tinted melanin-like pigment, for there seems to remain a faint suggestion of a dull-buff pigmentation in the palisade cells after the anthocyanin has been removed.

The basal-color layer is a strong orange yellow, the pigment being the melanin-like material usually found in this layer. This variety is the second generation of a cross of Red No. 4 on Whippoorwill No. 6.

No. 253–2–3B–23 is a cowpea having a general blue-black tint, due to a speckling of deep blue on a ground color of light or dark brown, the latter being more or less obscured by the darker color. In sections treated with neutral water this pigment, an anthocyanin, shows as a strong indigo blue, confined principally to the lower ends of the palisade cells. No trace of rose-red coloration was found. Decolorized sections, if stained with diamond fuchsin, show an intensity of stain in proportion to the degree in which the cells were pigmented, and it is then more clearly seen that a fair proportion of these cells, certainly more than one-half, are without this pigment. No indication of any melanin-like pigment is found in the palisade layer. With hydrogen peroxid and ferric sulphate the palisade layer is rapidly bleached, but the basal-color layer resists the

action of this powerful liquid for some time. This layer in water shows as a deep orange-buff color, due to the melanin-like pigment generally found in this layer.

The scheme of coloration is produced by the blue-black pigment above mentioned superimposed upon the strong-clay or light-coffee color found in the basal-color layer. The strong pigmentation around the hilum is identical in character with that found on the rest of the seed coat. This hybrid is the second generation of a cross between a blue Taylor No. 20 and a Red-Eye No. 26.

A very similar cowpea in general color scheme is the so-called blue Macassar No. 21299a. The seed coat ranges in various individual cowpeas from an almost purple blue to a complete black. Transverse sections of the seed coat show that the pigment, a blue black, is somewhat unevenly distributed throughout the palisade cells, although no cells seem to be absolutely destitute of it. Associated with this blue black is a small quantity of rose-colored pigment which occupies the upper part of the cell, the deep blue being uniformly found in the lower one-third. This rose-red pigment, an acid anthocyanin, is quite evenly distributed through the palisade layer, so that the inequality in the pigmentation of the seed coat, which may be detected with a hand lens, is due to the unequally deposited deep-blue, alkaline anthocyanin.

A mere suggestion of a faint buff pigment was detected in the palisade cells, presumably the melanin-like material found in this position in other cowpeas. But as the quantity, if present, is too small to play any rôle in the coloration and its presence in any quantity is unverified, it may be left out of consideration. It should also be stated that in this cowpea a test for tannin shows that an unusual quantity of some one of this group of compounds is present in the palisade cells.

The basal-color layer is strongly pigmented with the usual deep yellow melanin-like substance. The intense color superimposed upon this by the contents of the palisade layer probably prevents its having any considerable effect in the exterior coloration of the seed coat.

This variety was secured from Piracicaba, Brazil, May, 1907. There are no data on its parentage. It may be stated that in Brazil all varieties are called "macassar."

In No. 227–2–4, a black cowpea, all of the cells of the palisade layer are well filled, at least in the upper two-thirds, with a rose-red acid anthocyanin. Somewhat less than one-half contain also an extremely dense and granular indigo-blue alkaline anthocyanin. In cells destitute of the latter the rose anthocyanin fills the entire cell. Were it not for the intense coloration due to the rose anthocyanin the unequal distribution of the indigo-blue pigment would result in a blotched or speckled condition of the seed coat. The basal-color layer is a pale-straw tint or sometimes merely a cream white. The palisade cells are free from all con-

tortion or other modification of form. This hybrid is the second generation of a cross of Watson No. 5 on Coffee No. 27.

No. 228–5–4 is usually black, but shows great variability in its color scheme, ranging from uniform black into black with small irregula fawn-colored or reddish brown marbling, or a fawn and reddish brown marbled with black, or having blue-black speckles, or in rare instances the entire cowpea is a uniform fawn or light red brown, especially immature seeds. There are, therefore, three pigment elements to be considered: (1) Deep blue or black areas, (2) blue-black speckled areas, and (3) fawn to red-brown basal areas.

The deep-black areas show that this color is an intensification of a strong purple in the pigment cells, which, although it is present in all the cells of such areas, is still quite variable as to quantity, some cells being pigmented only in the extreme lower end and others through the entire cell cavity. On treatment with neutral water this color resolves into the two factors before noticed—namely, a rose-colored anthocyanin very uniformly distributed throughout this layer and an indigo-blue anthocyanin massed in the lower end of certain cells. It is therefore evident that these black areas would show a somewhat mottled condition if the excessive pigmentation did not obscure this.[1]

The second pigment element—namely, the blue-black speckling—shows a very different condition of things. This also is due to an anthocyanin deposit in the palisade cells, but it is clearly distinguishable from what was found in the solid black areas in four respects: (1) The color is always a vivid indigo blue, not a purplish blue, nor does it give a rosy diffusion in water; (2) the quantity of this blue pigment is very much greater in the cells producing the speckling than in those found in the solid black areas; (3) it always extends upward toward the top of these cells, instead of being segregated into a heavy mass at their base; (4) the cells containing this particular pigment are in small groups, not solid masses as in the black areas, but usually large enough to be seen by the unaided eye. This is the Taylor or New Era type of speckling.

The third and remaining color is that of the fawn or reddish brown basal tint, which is due to the usual melanin-like compound contained in the cells of the basal-color layer. It ranges from a faint yellow to an intense yellow or even a copper color. This variation in quantity is the cause of the difference in color of individuals, ranging from pale fawn to reddish brown.

The palisade cells are strikingly regular, straight in outline, narrow in diameter, and long. The entire seed coat is somewhat heavier than usual. The grandparents are Clay No. 17 crossed on Coffee No. 27.

[1] Prof. W. J. Spillman has informed the writer that among cultivated cowpeas these black areas occur only in hybrids having all the factors for black pigment and having also the factor for the Taylor or the New Era style of speckling, and also that such a type can not be fixed in cultivated cowpeas, although it is the normal condition in wild cowpeas.

No. 239–4–3–6 is what is known as a Holstein pattern, a cream-white basal color blotched with large masses of black. Transverse sections through the black areas show that the pigmentation scheme is similar to that already seen in the black areas of varieties above mentioned—namely, a rose-red anthocyanin filling the upper half or two-thirds of all the pigmented cells and a blue alkaline anthocyanin deposited in only a limited number of these cells. The cells destitute of this latter pigment are in small clusters of two to five and do not make up more than one-tenth of the colored areas of the pigment layer. Here, also, a mottling of the seed coat would result were it not obscured by the intense color obtained from the heavy pigmentation of this layer. In the basal-color layer we come to a variation that has not previously been observed. Portions of this layer underlying the heavily pigmented palisade cells, which give the black areas to the seed coat, are very heavily loaded with a dense yellow granular pigment, but where the basal-color layer underlies unpigmented or cream-white areas this pigment is either wholly lacking or consists of a mere trace. Where this pigment is present in large quantities it is massed in the upper cells of the layer and is a decided copper color, whereas the lower cells are, when the pigment is present at all, a pale lemon yellow.

A still more interesting departure from normal structure is found in the white areas of the palisade layer. In the black areas these cells are quite regular, both in form and in the gradual tapering of the cell cavity. But in the white or cream-white areas, although most of them are approximately regular, occasional cells, two or three together, show the strongly contorted form and the erratic spreading of the cell cavity, which were previously noticed in the albino cowpeas. In other words, in these cream-colored areas we find a duplication of the structure of the cells, as well as of color, that was discovered in certain cream-colored cowpeas previously described. Roughly estimated, 10 per cent of the cells of the white areas of this hybrid cowpea show this striking contortion in form and erratic spreading of the cell cavity. There is therefore seen to be a very strong contrast in structure, as well as in color, between the black and the white areas of this variety. This hybrid is the product of a Watson No. 5 crossed upon a Taylor No. 14, third generation.

Cowpea No. 227–5–1Re–17 has a color scheme of the Watson type. It is a pale-buff basal color, irregularly streaked with a purple black. The colored palisade cells are extremely dense with pigment, often appearing almost black. The quantity, however, varies greatly, some cells being practically pigment-free. This results in an irregular coloration of the seed coat. The pigment is an intense indigo-blue alkaline anthocyanin, with no trace of the rose-colored acid anthocyanin. There is also no melaninlike pigment in this layer. The cells are extremely irregular, the amount of contortion being greater than that of any variety previously mentioned. The cell cavity is also irregular, being

broadly flared out at the base, rapidly narrowing in the middle of the cell to a mere thread, and again broadening at the upper end. This irregularity of form should be noted in comparison with the same condition mentioned under other cowpeas belonging to this Watson type. In the basal-color layer large crystalline masses of a heavy melanin-like pigment are deposited in clustered cells along its upper stratum. Many of the cells are so scantily supplied with pigment as to appear practically colorless. The form of the cells is also more irregular than usual, showing a decided crumpling and contortion, even when expanded by means of caustic potash. This hybrid is the result of a cross of Sport No. 5 upon Coffee No. 27, third generation.

In Sport No. 5 the seed coat varies from cream white thinly spread over with purplish black spots arranged on the Watson type to individuals in which the purplish black pigment is so abundant as to give a dull purple-gray tone to the entire seed. All the cowpeas of this variety have a strong purplish black "eye." In the darker individuals the pigmented palisade cells, about one-fifth of the entire number, owe their color to an intense indigo-blue alkaline anthocyanin uniformly found in the lower third of the cells. The other four-fifths of the cells seem to be destitute of pigmentation. The somewhat purple tint of the seed coat would lead to the expectation that an acid anthocyanin would be associated with this blue anthocyanin, but no such color was discoverable. No trace of melanin-like pigment could be found in the palisade cells. Their shape is also significant. They are usually short, with thick walls, and display to a moderate degree the strange contortion in outline and twisting on their long axis referred to in the case of some other cowpeas. The basal-color layer is a pale or dirty lemon yellow.

The only difference discoverable between the light and the dark colored individuals is the greater infrequency of pigmented cells in the palisade layer of the former, there being only 1 in 20 having a trace of the blue-black anthocyanin above noted. It is also in smaller quantity. A mere hint of rose-red anthocyanin seems to be discoverable in palisade cells of the lighter individuals, but this is too uncertain to warrant a definite statement. The cells are also unusually short, thick-walled, and contorted in outline. The basal-color layer is the same as in the other form. No accurate data as to parentage of this variety were obtainable. It was originally secured from Mr. J. W. Trinkle, of Madison, Ind., but correspondence has failed to give the facts regarding its origin.

No. 220–2–2Re, another Watson type, has a pale-buff seed coat irregularly streaked with dull purple. The pigment is intensified around the hilum, producing what is known as an "eye." A minute amount of rose-red pigment is found in the upper part of the palisade cells. It quickly dissolves in water, leaving a dense mass of granular indigo pigment in the extreme basal end of the cell cavity. A

large part of the cells, however, are practically free from any color, this being the cause of the very irregular streaked appearance of the seed coat. The cells are extremely irregular in shape, which gives to a section through the palisade layer a marked unevenness of appearance. The basal-color layer has a pale dull-yellow pigment massed in its lower cells. A good number of the cells seem to be colorless. The variety is the second generation of a cross of a Watson No. 5 on a Coffee No. 16.

One other cowpea needs to be mentioned, No. 0618. It is pale buff or clay, dusted over with brown gray on the Watson pattern. It is further pigmented with very small, round, deep, purple-brown dots, similar to the color massed around the "eye." The pigmentation of the palisade cells is confined to a little over half their number, the pigment being in the lower third of the cell cavity. It is a blue alkaline anthocyanin, with little or no trace of the acid form of this pigment, although in the minute areas represented by the deep purple-brown dots of the seed coat a small amount of red acid anthocyanin seems to be present. All the palisade cells are strongly contorted. The underlying basal layer is narrow and also strongly contorted. It is largely destitute of pigment, except for segregated masses of a deep-orange color located in widely separated groups of cells in the upper portion of the layer. The parentage of this hybrid is unknown. Like No. 5, previously mentioned, it was obtained from Mr. J. W. Trinkle, of Madison, Ind., but correspondence has failed to give any data regarding its origin.

III.—COWPEAS HAVING ONLY A MELANIN-LIKE PIGMENT IN THE PALISADE LAYER

The third class of cowpeas—namely, those in which a melanin-like pigment alone is found in the palisade layer—ranges through various shades of light brown, buff, and red.

The first of these is a pale-buff or clay-colored cowpea, No. 237–3–7. A minute quantity of melanin-like pigment was detected in the palisade cells, and that only in the case of darker specimens. The very pale buff-colored seeds show no trace of pigmentation in this layer. The effect of this minute trace of pigment on the general color scheme of the seed coat must be small. Indeed, the color is easily explained by the stronger pigmentation of the basal-color layer. This layer is a vivid brownish yellow color. All the tests for anthocyanin failed to show a trace of this prevailing pigment in any cells of this cowpea.

It should be noted that the presence of melanin-like pigment in the palisade cells is of some interest in regard to the affinities of this cowpea to others in which it is also found, its parentage being Red No. 4 crossed on Taylor No. 14, second generation from the cross.

Another practically monochrome cowpea is No. 27544, known as the Iron cowpea. It ranges from a delicate buff or clay to a strong reddish

brown with an intensification of color about the hilum. A part of the palisade cells, perhaps two-thirds to four-fifths, contains a moderate quantity of the melanin-like pigment, the remaining cells being pigmented to only an extremely slight degree. The pigment is scattered throughout the cell cavity in a fine granular condition, instead of being massed in the lower end, as is usually the case. The basal-color layer ranges from a strong yellow to a decided copper or orange color, varying in this respect according to the general coloration of the seed coat itself. It has been impossible to learn the parentage of this well-known and widely cultivated variety.

A cowpea strongly marked in what is known as the Whippoorwill pattern, made up from a basal color of a pale clay heavily marbled with a rich reddish brown, is No. 242–3–1. The palisade cells show the variation in coloration that would be expected by the marbled character of the seed coat. The strongly pigmented cells of the marbled areas are a rich reddish brown, approaching to the color found in the basal-color layer. The other cells, making up the unmarbled areas, though not actually destitute of pigmentation, contain so minute a quantity as to only slightly affect the color of the basal layer beneath it. This latter layer is of an intense copper tint, the pigment being deposited in dense masses in the upper part of the layer. A very unequal distribution of the pigment in this layer corresponds somewhat but not accurately to the unequal distribution of the pigmentation in the overlying palisade layer. No trace of anthocyanin was found in any of the cells of this cowpea. Although the optical effect in the matter of color is not involved in the presence of tannin, it may be stated that this substance is more abundant than usual in this particular cowpea. Its parentage is Clay No. 17, crossed on Whippoorwill No. 6, second generation.

No. 243–5–3 is a variety with monochrome seed coat ranging in color from a light to a very dark reddish brown. The cells of the palisade layer show a strong granular pigment of a light red, in some cases almost brick red, quite uniformly massed in the extreme lower end of these cells. In some instances the pigment is so finely divided that it is difficult to discover it except when masses of cells are superimposed upon one another. Although the seed coat gives no indication of an unequal distribution of color, the sections seem to indicate that there is a slight excess of pigment in certain groups of cells over that in cells surrounding them. The basal-color layer has a much lighter tint than that found in the cowpea last mentioned. It is a lemon-yellow color, intensified in darker individuals to a decided brassy tone. The form of the palisade cells is normal. The parentage of this cowpea is a Red No. 4 crossed on a Whippoorwill No. 6, second generation.

No. 242–5–2 has one of the two parents last mentioned and is similar in general color scheme, varying from buff to reddish brown. The palisade cells are abundantly supplied with a dull-yellow pigment, but quite vari-

able in quantity. In view of the fact that one of its parents is a Whippoorwill this unevenness of distribution of pigment in the palisade layer is significant. The cells of this layer are longer than usual and the taper of the cell cavity is somewhat sudden and blunt. There is, however, no contortion. There is no evidence of anthocyanin. The color in the basal-color layer is somewhat different in tone from that in the palisade layer, being a more vivid yellow, approaching orange; but both give reactions that indicate the pigment to be the usual melanin-like substance. Tests for tannin show that the basal-color layer is highly impregnated with this substance. The variety is a cross of Whippoorwill No. 6 on Clay No. 17.

No. 216–6–4, a light-coffee cowpea obscurely streaked, shows the basal-color layer to be a vivid yellow, while the palisade layer is buff to brown and quite variable in degree of pigmentation. The pigment in both is a strongly granulated melanin-like substance. There is no trace of anthocyanin. The cowpea is a second-generation hybrid produced by crossing Red No. 4 upon Coffee No. 16.

No. 216–1–7 is a light to dark coffee cowpea. Closely observed, the seed coat shows a slight tendency to mottling. The very decided color of the seed coat would lead one to expect a heavy pigmentation in the palisade layer, but such is not the case. It is pale reddish brown, and not only is comparatively light, but treatment with various reagents fails to produce much intensification. In the basal-color layer the pigment is far more abundant and is confined to three or four layers, where it is somewhat unevenly distributed. It seems that the deep red brown of this cowpea is due to the pale reddish brown of the palisade layer plus the intense orange yellow of the basal layer. The parents are Red No. 4 crossed upon Coffee No. 16; in other words, it is identical with those of the cowpea last mentioned, the variety examined being the second generation of this cross.

A most interesting cowpea, known as Old Man, bears the Government number 17354. It has a cream-white seed coat obscurely and faintly streaked with yellow brown. The deeper color is very strongly deposited about the hilum, so that its character can there be readily tested. Transverse sections of the seed coat show that in almost all instances the palisade cells are practically destitute of pigment. However, a minute quantity may be detected by very close observation, and it is observable that this is highly variable, even within the narrow limits just mentioned. In other words, it corresponds to the very obscure streaking of the seed coat itself. It is of a melanin-like character without any admixture of anthocyanin. The palisade structure is decidedly abnormal, its cells being much wider in proportion to their length than common, enormously contorted, and the unusual twisting upon the long axis is here carried to an extreme. The whole palisade layer is loosely put together with abundant intercellular spaces. The basal-color layer has an exceedingly meager

and pale representation of the pigment usually present. The pale-cream color of this cowpea is doubtless due to the small quantity of melanin-like pigment diffused through the basal-color layer, and the streaked and indistinct marking of the seed coat is caused by the minute quantity of the same pigment unevenly distributed in the palisade layer. It is interesting to note that the palisade cells in the neighborhood of the hilum, where the color is quite intense and forms what is known as the "eye," are very much larger than on the rest of the seed coat and almost entirely free from the contortion and twisting already mentioned. In other words, the irregularity of form seems to be directly connected with the white or cream-white character of the seed coat. This same remarkable parallel has already been noted in several other cowpeas. The basal-color layer in the neighborhood of the hilum is very heavily charged with a melanin-like pigment, but there is here a somewhat unusual arrangement in that the lower cells of this layer are of a somewhat pale lemon yellow, while the separated masses in the upper part of the layer are a deep orange or orange buff. The reactions of these two, however, are identical. No information has been obtained as to the parentage of this variety.

IV.—COWPEAS HAVING BOTH A MELANIN-LIKE PIGMENT AND ANTHOCYANIN IN THE PALISADE LAYER

The fourth class includes all cowpeas showing both anthocyanin and melanin-like pigment in thepa lisade layer. The first one to be mentioned, No. 214–5–10, is generally described as having buff markings upon a black ground. The fact is that it is a cowpea with a strong buff basal color almost covered with large black areas. In other words, the black is superimposed upon the buff and not the buff upon the black. The two colorations of the seed coat are accompanied by a quite different condition of the palisade layer. A melanin-like substance is to be found in all pigment cells of the seed coat both in the buff and in the black areas. An acid anthocyanin is present in all the palisade cells of the black areas, but in no case in those of the buff areas. An alkaline anthocyanin is to be found in one-half to three-fourths of the palisade cells of the black areas, but in none of the cells of the buff areas. In all cases the alkaline anthocyanin is massed in the lower end of the cell cavity and the acid anthocyanin occupies principally, if not wholly, the upper half of the cell cavity. The color produced by these two anthocyanin pigments is a more intense purple than has been found in any other cowpea, and when the rose colored acid anthocyanin is extracted, the indigo-blue or alkaline anthocyanin found in one-half to three-fourths of the cells of the black areas is larger in quantity and more vivid in color than is generally the case. In the buff areas there is evidently neither of these phases of anthocyanin. These cells are, however, pigmented with the melanin-like material found in other cowpeas. A comparison of the form of the palisade cells in the two areas is also of interest. Those in the black

areas are unusually symmetrical, so much so as to attract attention, but in the buff areas there is a slight tendency to contortion and a more unequal tapering of the cell cavity. In other words, there is a hint in these cells of the abnormality of form found in a high degree in some other cowpeas. The basal-color layer is well supplied with the usual yellow melanin-like pigment in all parts of the seed coat. The parentage of this cowpea is White No. 7 crossed upon Black No. 22, it being the second generation hybrid.

A cowpea that appears in general purplish black, but somewhat unevenly colored, is No. 201–1–2–9. A study of its seed coat makes the cause of this evident. Many of the palisade cells contain only one anthocyanin pigment—namely, a strong rose purple. This dissolves rapidly in water, leaving the cells colorless. In some cases a second color remains in the cells and proves to be minute particles of the usual melanin-like pigment. In addition to the foregoing a number of the cells contain in the lower end a strong deposit of blue alkaline anthocyanin. This is more clearly seen after the extraction of the rose-colored anthocyanin. The melanin-like pigment is unevenly distributed in the palisade layer, many of the cells being destitute of it, so that it is safe to state that in some areas of this cowpea this pigment is associated with both phases of anthocyanin while in other parts we have either the rose anthocyanin alone or the rose and the indigo-blue phases of this pigment without the presence of the melanin-like pigment. The cross producing this variety is Black No. 13 upon Blackeye No. 19, being the third generation from the cross.

Although No. 239–5–3–18 was referred to under the first division as being an essentially cream-white cowpea, the strong purple eye of this variety places it in this last division; for by making transverse sections in the neighborhood of the hilum where the pigmentation is intense we find that the palisade layer contains both the acid and the alkaline phase of anthocyanin associated with the melanin-like pigment. The rose, acid anthocyanin is quite generally present in these pigmented cells, but a large number of them, perhaps two-thirds, are destitute of alkaline anthocyanin. The basal-color layer is abundantly colored with the usual orange-yellow pigment. As stated, the palisade cells in the cream-white seed coat, which constitutes almost the entire surface of this cowpea, are unusually irregular in form. It is therefore quite interesting to see that the strongly pigmented palisade cells in the neighborhood of the hilum show no trace whatever of these irregularities. As already stated, the hybrid is the third generation of a cross between Watson No. 5 and Taylor No. 14.

A cowpea having a basal color ranging from pale buff to strong red brown and very heavily spotted with black is No. 214–6–7–2. There is seen to be a strong brassy yellow pigment in the palisade cells. The basal-color layer is usually densely filled with the same colored pigment,

but where the basal color is pale buff instead of red brown it is very deficient. The deeper tint of the basal color in some seeds is therefore due, at least in part, to a greater quantity of pigment in the basal-color layer rather than to any difference in the pigmentation of the palisade layer. This is the reverse of what is usually found in cowpeas of variable tint, their difference usually being brought about by variation in the degree of the pigmenting of the palisade cells superimposed upon a uniformly pigmented basal layer. The black areas are due to a dense blue alkaline anthocyanin confined to the lower third of the cavity of the pigmented cells and so heavily deposited that only long action by different reagents brings about the usual changes. The brassy yellow pigment also contained in the palisade cells gives the usual reactions. It is strongly granular and in unusually large quantity. It is well to note the presence in the same cells of these two forms of pigment in connection with the fact of the very slow response of the anthocyanin to the usual reagents, as this behavior will be commented upon in other cases. The form of the palisade cells is slightly irregular, but not exceedingly so, and this is confined almost entirely to the lighter portions of the seed coat. The parentage is White No. 7 crossed upon Black No. 22, second generation.

Cowpea No. 14 has a pale-buff to red-brown basal color, strongly speckled with black spots on what is known as the Taylor pattern. In individuals of the lighter basal color we find that the palisade cells in the ground-color areas are so nearly destitute of pigment that it is difficult to discover its presence. The basal-color layer is also a dirty yellowish brown instead of the stronger brown that would be expected. The areas spotted with black owe this color to an intense blue anthocyanin in the lower half of the cavity of the palisade cells, the proportion of these to uncolored cells being about as 1 to 5. The anthocyanin extends up in these cells much higher than in most cases, sometimes reaching the upper end of the cavity. In individuals having the darker ground color, the presence of melanin-like material in the palisade cells is very evident and the basal-color layer is seen to be much more strongly tinted with a strong copper-colored melanin-like pigment. In this cowpea we again find that only by long treatment will the usual reagents bring about the expected reactions on the anthocyanin. Caustic soda, hydrochloric acid, chloral hydrate, etc., are very sluggish in the changes produced, so that there seems to be an impediment in the way of their reacting upon this sensitive material. The palisade cells in both dark and light varieties are long, narrow, evenly tapered, and symmetrical. No data have been secured as to the parentage of this variety.

No. 237–3–2 also ranges from pale buff or clay to strong red brown and is speckled with black. The black color is due to a blue alkaline anthocyanin deposited as usual in the lower end of the palisade cells, and in this case also the reagents are extremely slow in producing results

upon this pigment. This fact is of interest when taken in connection with the fact found in the other cowpeas above mentioned that it is intimately associated in these cells with a very large amount of melanin-like pigment.[1] In what way the intimate mixing of these two protects the anthocyanin from the rapid effect of reagents it is impossible to say, but it seems probable that some such interference is brought about. The melanin-like pigment is coarsely granular and orange brown in color. It is also to be observed that this pigment modifies the color tone tardily secured by the reactions of various reagents. Thus, with hydrochloric acid, the blue anthocyanin does not give a rose color, but rather a deep cherry red, probably due to the mixture of the usual rose tint with the orange yellow tint of the melanin-like pigment associated with it. The speckling, which is of the Taylor type, is due to anthocyanin contained in certain palisade cells. The basal-color layer has the usual pigment. The cowpea is the product of a cross of Red No. 4 upon Taylor No. 14, second generation.

Another cowpea of a different color scheme needs mentioning, No. 243-6-1. This one ranges from pale buff to strong red brown, speckled with black, on the New Era pattern. The palisade cells which represent the ground color—that is, which are not connected with the speckling—are, as in the former case, of a dull brassy yellow. The same tint is found in the basal-color layer. From one-third to one-fifth of the palisade cells contain a deep-blue alkaline anthocyanin located in the lower end, and here again it was discovered that all the reactions normal to this pigment are greatly delayed, so that a longer period of time is needed to make the necessary tests. The melanin-like pigment is present in large quantity in all the palisade cells. This variety is the second generation of a cross of Red No. 4, crossed upon Whippoorwill No. 6.[2]

We come now to a cowpea which is probably wild. It is a *Vigna sinensis* (?), having the number 01653, and comes from Sokoto Province, Upper Nigeria, Africa. In some respects it is quite different from the cultivated cowpea. In matter of size it is from one-seventh to one-eighth the average size of cultivated varieties. Its markings are extremely interesting, in that they display on the same seed coat all of the features which are found to make up the color schemes of the cultivated cowpeas, not only all the colors but all the styles of distribution. First, there is a basal color which ranges from pale clay or buff to reddish brown; second, this is extensively blotched or marbled with deep brown red, sometimes pretty well covering the seed coat; third, there is present a fine speckling of blue-black dots scattered over the seed coat; and

[1] This intimate mixing of blue anthocyanin with a deep-tinted melanin-like pigment and the consequent resistance of the former to reagents misled the writer at first into concluding that he here had to do with a black melanin-like substance; and in some remarks before the Washington Botanical Society on May 7, 1912, the writer included such pigment with the others found in the cowpea. A report of this meeting, in Science, June 28, 1912, also contains this error, which is now corrected.

[2] Some individuals of this variety proved to have been contaminated by crossing; hence, the presence of speckling in some of its descendants.—W. J. SPILLMAN.

fourth, there are occasional spots in the form of large roundish intensely black areas. Transverse sections show that the general structure of the seed coat is identical with that of cultivated varieties. The palisade cells are of the same general shape and are as to size in the usual proportion to the rest of the seed coat. The underlying layer of so-called hourglass cells is also the same and is, as elsewhere, empty. Beneath this is the usual basal-color layer, supplied with the regular orange or yellow melanin-like pigment. The red areas of the seed coat overlying the basal clay or buff owe their color to a strong orange or red-brown pigment in the palisade cells, identical in organization and in its reactions with the similar color in cultivated varieties; in other words, a melanin-like pigment. The fine speckling is in this case also due to an intense blue anthocyanin pigment in the lower end of certain palisade cells and it is also here associated with the melanin-like pigment mentioned under former headings, and, as in the other cases, it responds very slowly to the reaction of reagents. Furthermore, the areas represented in the seed coat by large black spots contain both red acid-anthocyanin and blue alkaline anthocyanin, as is the case in the black areas of cultivated cowpeas. The complete uniformity of methods of coloration, as well as of schemes or patterns of coloration in this supposedly wild cowpea, when compared to our cultivated varieties, is of considerable interest. There is no trace of distortion or irregularity in the palisade layer. Of course, no knowledge is obtainable as to its origin. It was received from Kew Herbarium and was collected by J. M. Dalziel.

SUMMARY

The greatly diversified color schemes of the different varieties of cowpeas may therefore be reduced to two factors: (1) An extremely uniform basal color, ranging from very pale yellow to deep copper red, but found to be in all cases due to a melanin-like pigment deposited in the basal-color layer, the differences in tint being unquestionably caused by differences in quantity rather than in character of the pigment present; and (2) a superimposition upon this basal color of variously arranged pigment areas in the palisade layer, the outer layer of the seed coat, the pigments here being of only two kinds, first, a melanin-like pigment very generally identical in color and behavior to that found in the basal layer, and, second, an anthocyanin pigment, either associated with this or found in separate cells. And further, this anthocyanin pigment may be of a red color, on account of an acid condition, thereby producing various shades of purple and rose; or it may be alkaline in character, thereby producing various shades of blue and black, and these two may be found in the same cells or in some instances in separate cells. Finally, according as only one, or more than one, or all of these pigments sometimes found in the palisade layer are actually present there, and according as they are uniformly distributed throughout its cells or are variously localized in large or small

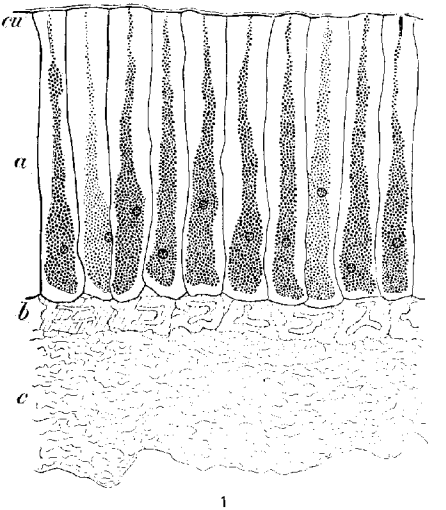
areas of its cells, do we get the remarkably diversified blotching, streaking, speckling, marbling, or monochrome colorations which characterize the different varieties of cowpeas.

A word should be said regarding the very interesting cases of distortion in the palisade cells mentioned under some of the foregoing varieties. Referring to the facts there mentioned, it will be seen that where the seed coat of the cowpea is white or cream white, as in Nos. 0362 or 17354, or where it has a certain white area, as in Holstein No. 239–4–3–6 or in No. 239–5–3–18, or even in cases where there is merely a light speckling or dusting over of this cream-white color, as in Sport No. 5, in varieties of the Watson type, as No. 227–5–1 Re–17, in certain individuals of No. 17354, and in No. 0618, the palisade cells show great distortion of outline and unevenness in the cell cavity. Furthermore, in most particolored cowpeas of strongly contrasted tints, such as Holstein No. 239–4–3–6, or the black eye in No. 239–5–3–18, or the coffee-colored eye in No. 17354, the strongly colored areas have perfectly regular, symmetrical palisade cells, while the lighter areas are more or less strongly contorted in form and irregular in the cell cavity. In other words, there is traceable in all of these cowpeas a decided correlation between the morphology of the palisade cells and the suppression of the pigments in these cells.

PLATE VI

Fig. 1.—Transverse section of the seed coat of a cowpea, similar to that shown in text figure 1, but showing the relative thickness of three layers, as on the seed. The cells are not expanded with chloral hydrate. *cu*, Cuticle; *a*, palisade layer; *b*, middle or hour-glass layer; *c*, basal-color layer. Somewhat diagrammatic.

Fig. 2.—Seeds of cowpeas, showing some of the variations in the style of marking of the seed coat. Natural size.



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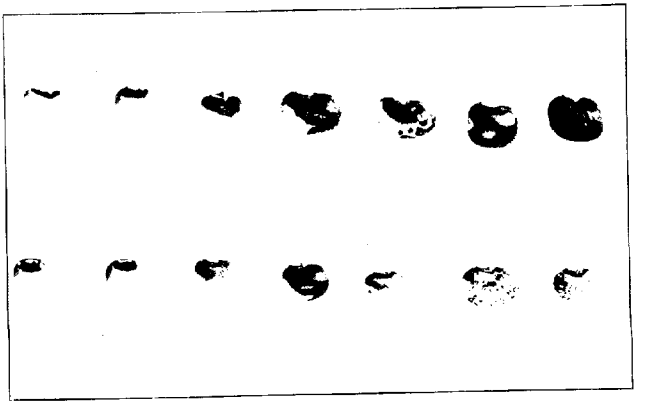

2

PRELIMINARY AND MINOR PAPERS

EXPERIMENTS WITH APPLE LEAF-SPOT FUNGI

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INTRODUCTION

That *Sphaeropsis malorum* is the organism which causes the common leaf-spot of the apple (*Malus* spp.) so prevalent in the East and South was first shown by Scott and Rorer (1907).[1] The work of these investigators was later confirmed by the experiments of Brooks and De Meritt (1912), Lewis (1909, 1912), and others.

In various parts of the South—in Virginia, West Virginia, North Carolina, Kentucky, and Tennessee, and probably in other States—this leaf-spot, usually so prevalent in the spring, later enlarges and becomes a harboring place for various species of fungi. Such enlargements, which give to the disease the common name of "frog-eye," are usually in alternating rings or zones of brown and gray. Sometimes they form complete circles concentric with the original spot, but more often they are only half circles whose centers lie near its margin (Pl. VII, fig. 1). Around these enlargements others may be formed until perhaps one-third of the leaf is involved.

Hartley (1908) found that enlargement of spotted or injured areas could be induced to a slight extent by *Coniothyrium pirinum*. Sheldon (1908) considers the frog-eye disease in West Virginia to be due to *Illosporium malifoliorum* because of its association with the disease. Crabill (1913) gives an excellent description of the disease and expresses the belief that *Phyllosticta pirina* is very probably the factor to which the rings are due, the original spots being caused by *Sphaeropsis malorum*. The organism, however, which Crabill calls *Phyllosticta pirina*, or at least his strain No. 2 with pink spore masses, the writer believes to be *Phyllosticta limitata*. At any rate, since *Phyllosticta pirina* is a synonym of *Coniothyrium pirinum*, to recognize the two names as applying to two different fungi, as Crabill does, would be an obvious error. The fungus *Phyllosticta pirina* was transferred by Sheldon (1907) to the genus Coniothyrium on account of the color of its spores, thus making *Phyllosticta pirina* Sacc. a synonym of *Coniothyrium pirinum* (Sacc.) Sheldon. The fungus hereafter mentioned as *Phyllosticta limitata* has pink spore masses on certain culture media and on apple leaf-spots. In cultural characters it corresponds to Crabill's *Phyllosticta pirina*, strain No. 2. Its spore measurements average 7 by 4 microns.

THE FUNGI

The writer undertook to determine whether certain leaf-spot fungi were capable of enlarging spots already formed. In the beginning the following fungi isolated from zoned spots were employed: *Coniothyrium*

[1] Bibliographic citations in parentheses refer to "Literature cited," p. 65.

pirinum, *Coryneum foliicolum*, *Phyllosticta limitata*, *Monochaetia mali*, *Phomopsis mali*, and a species of Pestalozzia. Later, a species of Alternaria was also employed, to which the name *Alternaria mali* will be given, though, owing to the confused state of names and descriptions in this genus, the identification of this species with any degree of certainty is impossible. The description of the fungus and of its appearance when growing on various artificial media serves to separate it from the two or three other species which occur on the apple. Its cultural characters alone serve to separate it from one of these species, and it differs from all of them in the possession of a minutely spiny or nearly verrucose exosporium. The mycelium, as is common in the genus Alternaria, is composed of olive-tinged hyphæ rather sparingly branched. The club-shaped spores (Pl. VII, fig. 2) are for the most part muriform-septate, the transverse septa numbering usually from three to five in mature spores. The spores are 30 to 35 by 12 to 13 μ in size, with isthmi measuring 4.5 to 7 by 3 to 4 μ. Its technical description is as follows:

Alternaria mali, sp. nov.—Hyphis fasciculatis septatis subsimplicibus vel ramulosis, griseo-olivaceis; conidiis clavatis, olivaceo-brunneis, 3–5 septato-muriformibus, ad septa constrictis, breve hispidis, 30–35×12–13 μ, isthmis 4.5–7×3–4 μ.

Hab. In foliis *Pyri mali*, Arlington, Virginia.

The growth of *Alternaria mali* on culture media was as follows:

BEEF AGAR +10.—Growth diffuse, grayish to nearly black at surface; aerial hyphæ nearly white and rather short.

BEEF BOUILLON.—Growth much as on beef agar, forming compact disk over surface of liquid; brownish where in contact with liquid and nearly white above.

PRUNE JUICE.—Growth forming disk over surface of medium, greenish black below; abundant flocculi of rather long aerial hyphæ, which are gray, slightly tinged with green.

PRUNE AGAR.—Growth very abundant, forming dark, nearly black crust over surface of slant, with abundance of rather long, greenish gray, flocculent aerial hyphæ, becoming darker green near the surface.

CORN-MEAL AGAR.—Growth the same as on prune agar.

BEAN PODS.—Growth black where in contact with tube and dark gray where in contact with liquid. Aerial hyphæ fairly long over pod and from light gray to nearly white.

POTATO AGAR.—Growth nearly black at surface of media, with very short, scant, gray aerial hyphæ. Greatly resembles growth on beef agar.

Alternaria mali has several times during the last year been isolated from fruit of the apple by Mr. D. F. Fisher, of the Office of Fruit-Disease Investigations, and has been found by inoculation to cause a rapid rotting of ripe apples.

Sphaeropsis malorum was also used in some of the later experiments.

Lewis (1909, 1912) has shown that at least in Maine the first three of these fungi are saprophytes capable of growing and fruiting only on spots previously killed.

EXPERIMENTS AND OBSERVATIONS ON THE FUNGUS

Inoculations were made both in greenhouse and in orchard on unsterilized leaves of susceptible varieties of apples, chiefly the Ben Davis and the York Imperial. Circular dead spots were made by touching the leaves with the heated end of a cylindrical steel rod 2 mm. in diameter. About 2,000 inoculations were made by spraying each spot with distilled water containing spores. Spots sprayed with distilled water only were considered as checks. The leaves were kept moist for periods of from one

to seven days either by inclosure in paper bags or by beginning the experiments during an extended rainy period. It soon became evident that zonate enlargements were developing from some of the check spots as well as from some of the inoculated ones, and in nearly every case the species of Alternaria appeared in culture from these enlargements. It was suspected, however, that the Alternaria was only a saprophyte and that water standing on the leaf for some time and soaking into the dead spot had thence reached the intercellular spaces of the adjacent living portion of the leaf and caused death by preventing gaseous exchange. Accordingly it was thought that if a branch of a susceptible variety with spots burned on its leaves were placed under very moist conditions the zoned areas could be induced quickly and abundantly.

On July 8 a branch of the York Imperial variety having sound leaves was selected and the cut end placed in a flask of distilled water. Without previous washing or sterilization the leaves were scorched with the heated end of the steel rod. The branch was then drenched with distilled water and placed under a bell jar partly lined with wet filter paper. Twice during the experiment the branch was taken out, and after the water on the leaves had been allowed to evaporate it was again drenched and placed under the bell jar. Under these conditions the leaves of the York Imperial apple held up as well as if they had been on the tree, there being no noticeable etiolation. In nine days 86 spots out of 111, or 77 per cent, had enlarged zones from 1 to 4 mm. in diameter, there being two or three zones to each spot. Such enlargements were then cut out from the leaves, dipped for a moment in 95 per cent alcohol, in mercuric-chlorid solution (1 to 1,000) for two minutes, in sterile water for five minutes, and then placed upon culture media. Of these cultures 17 out of 21 developed the Alternaria and 4 were sterile.

On July 19 this experiment was repeated, with the addition of two others, as follows:

EXPERIMENT 1.—Healthy leaves on branches of York Imperial apples were scorched with the heated end of a steel rod, sprinkled with distilled water, and placed under a bell jar lined with wet filter paper.

EXPERIMENT 2.—Treatment was the same as in No. 1, except that the branches were first immersed in 95 per cent alcohol for a moment, then in mercuric-chlorid solution (1 to 1,000) for two minutes, and washed in sterile water for five minutes.

EXPERIMENT 3.—One branch was treated as in No. 1 and one as in No. 2, but neither was placed under a bell jar or kept moist in any way.

The results after nine days are summarized in Table I.

TABLE I.—*Results of inoculation experiments of July 19 on branches of York Imperial apples.*

Experiment No.	Number of burned spots.	Number of enlarged spots.	Average width of enlargements.	Percentage of enlarged spots.
			Mm.	
1	71	51	7	72
2	44	3	3	7
3	39	0	0	0

Cultures were made as in the previous experiment, except that pieces of material were left in the mercuric-chlorid solution for three minutes. Of these 9 out of 17 developed the Alternaria, 7 contained bacteria, and 1 was sterile. In these experiments it will be seen that sterilization of the leaf surfaces practically prevented the disease.

On July 28 a series of four experiments was begun. Experiments 1, 2, and 3 were performed in the same manner as Nos. 1, 2, and 3 of July 19. Experiment 4 was performed in the same manner as experiment 2, except that after the washing in sterile water the leaves were sprayed with water containing Alternaria spores from cultures made from the spot enlargements of the experiment of July 8.

In three days some of the spots were beginning to enlarge, and in seven days the results were as shown in Table II.

TABLE II.—*Results of inoculation experiments of July 28 on York Imperial apple branches.*

Experiment No.	Number of burned spots.	Number of enlargements.	Percentage of enlarged spots.
1	101	66	65
2	94	8	9
3	41	0	0
4	82	46	56

Of 15 reisolation cultures from experiment 4, 12 developed Alternaria in pure culture and 3 contained only bacteria. Out of 18 cultures from spot enlargements of Nos. 1 and 2, 15 contained pure cultures of the Alternaria and 3 were sterile.

No counts of spot enlargements were made in the experiments of August 5, but it was noted that in those cases in which leaves were treated with alcohol and with mercuric-chlorid solution or with the mercuric-chlorid solution alone there were considerably fewer enlargements than in those untreated or those sterilized and inoculated with Alternaria spores or treated with alcohol alone. All the branches were placed under bell jars lined with wet filter paper.

The experiments of August 9 were carried on as follows:

EXPERIMENT 1.—York Imperial apple branches were placed in 95 per cent alcohol for a moment, in mercuric-chlorid solution (1 to 1,000) for three minutes, and in sterile water for five minutes. Spots were then burned on the leaves, and, after being drenched with sterile water, the branches with their cut ends in a flask of water were placed under a bell jar lined with wet filter paper.

EXPERIMENT 2.—Same as experiment 1 except that treatment with mercuric-chlorid solution was omitted.

EXPERIMENT 3.—Same as experiment 1 except that immersion in alcohol was omitted.

EXPERIMENT 4.—Treatment was the same as in experiment 1, but in addition the leaves were sprayed with sterile water containing spores of Alternaria isolated from enlarged spots of experiment of July 8.

EXPERIMENT 5.—Spots were made with a heated rod, as in the other experiments, and the leaves drenched with sterile water. The branches were then placed under a bell jar lined with wet filter paper, as in the preceding experiments. The results are given in Table III.

TABLE III.—*Results of inoculation experiments of August 9 on York Imperial apple branches.*

Experiment No.	Number of burned spots.	Width of enlargements.	Number of enlarged spots.			Percentage of enlarged spots.		
			Aug. 14.	Aug. 16.	Aug. 18.	Aug. 14.	Aug. 16.	Aug. 18.
		Mm.						
1	216	2 to 4	15	15	15	7	7	7
2	162	2 to 4	9	40	77	6	25	48
3	215	2 to 4	10	17	26	5	8	12
4	192	2 to 4	81	81	162	42	42	84
5	105	2 to 4	38	38	61	36	36	58

Cultures were made from enlarged portions of spots from experiments 2, 4, and 5. In each case marginal parts of the dead tissue were cut out, placed for a moment in 95 per cent alcohol, then in mercuric-chlorid solution (1 to 1,000) for three minutes, and in sterile water for five minutes. Of 11 cultures from experiment 2, 6 developed

Alternaria, 3 contained bacteria only, and 2 were sterile. Out of a total of 15 cultures from spot enlargements of experiment 4, 11 contained Alternaria and 4 developed bacteria. Out of a total of 12 cultures from experiment 5, 11 contained Alternaria and 1 developed bacteria.

On August 19 the following experiments were begun:

The material used in experiments 1, 2, 3, and 4 was sterilized as in No. 1 of the series of August 9. Circular spots were burned on all leaves, as in previous experiments. Nos. 1, 2, and 3 were sprayed with distilled water containing spores of *Coniothyrium pirinum*, *Phyllosticta limitata*, and *Alternaria* sp., respectively. Experiment 4 corresponded to No. 1 in the experiments of August 9 and was regarded as a check. The material used in experiment 5 was not sterilized nor inoculated, but received a thorough drenching. The branches with their cut ends in flasks of distilled water were then placed under bell jars lined with wet filter paper. The results are given in Table IV.

TABLE IV.—*Results of inoculation experiments of August 19 on York Imperial and Ben Davis apple branches.*

YORK IMPERIAL.

Experiment No.	Number of burned spots.	Number of enlarged spots.	Average width of enlargements.	Percentage of enlarged spots.
			Mm.	
1	86	12	1.5	14
2	69	7	1.5	10
3	81	40	3	49
4	63	3	3	5
5	32	23	3	72

BEN DAVIS.

1	32	12	2	38
2	32	12	1.5	38
3	31	30	3	97
4	30	1	3	3
5	43	25	2	58

Many of the enlargements in experiments 1 and 2 were somewhat doubtful ones, especially those on Ben Davis; in these two experiments the fungi fruited on practically every spot, but no fruits were found on any of the enlarged portions. Fruits would appear up to and in fact were most frequently found at the very margins of the original spots, but in no case did they occur on the newly formed parts. This agrees with the observations of Hartley (1908), who found the margins of spots to be the favorite fruiting places of *Coniothyrium pirinum*.

In cultures made as in previous experiments, material from experiment 1 produced 6 growths of the Alternaria and 2 of bacteria, while the remaining 1 was sterile; of 7 cultures from experiment 2, 2 developed Phyllosticta, 1 contained bacteria, and 4 were sterile. Out of 8 cultures from experiment 3, 6 developed Alternaria and 2 contained bacteria. Out of 15 cultures from experiments 4 and 5, 11 developed Alternaria, 2 contained bacteria, and 2 were sterile.

Experiments begun August 30 were carried on as follows:

Experiments 1, 2, 3, and 4 were performed in the same manner as those of August 19. The leaves in experiment 5 received the same treatment as those in experiment 4, but in addition they were sprayed with water containing spores of *Phomopsis mali*. As before, the branches were placed under bell jars lined with wet filter paper. The results are given in Table V.

TABLE V.—*Results of inoculation experiments of August 30 on York Imperial and Ben Davis apple branches.*

Experiment No.	Number of burned spots.	Number of enlarged spots.	Average width of enlargements.	Percentage of enlarged spots.
			Mm.	
YORK IMPERIAL.				
1	115	13	1	11
2	93	2	1	2
3	102	19	1.5	19
4	65	0	0	0
5	117	2	1	2
BEN DAVIS.				
1	50	18	1.5	36
2	29	0	0	0
3	34	14	3	41
4	51	6	2	12
5	43	2	2	5

Hyphæ and spores of Alternaria were found on both of the spot enlargements of York Imperial apple experiment 5.

The experiments of September 3 were carried on in an orchard at Arlington, Va. The trees selected were 10-year-old York Imperials which were entirely free from foliage diseases. These trees were planted quite closely together, so that certain portions were protected from direct sunlight during the afternoon. Leaves partly shaded in this way were selected as the most favorable for successful inoculation.

EXPERIMENT 1.—The leaves were spotted with a heated rod, as in previous experiments, and without previous sterilization were thoroughly sprayed with distilled water containing thick masses of spores of Alternaria obtained from cultures from the spot enlargements in the experiments of July 19.

EXPERIMENT 2.—The leaves were spotted as in No. 1 and without sterilization were sprayed with distilled water.

The weather during September was quite dry, but rains occurred with great frequency during October.

The results on October 22 are shown in Table VI.

TABLE VI.—*Results of inoculation experiments of September 3 on York Imperial apple branches.*

Experiment No.	Number of burned spots.	Number of enlarged spots.	Average width of enlargements.	Percentage of enlarged spots.
			Mm.	
1	127	71	1	56
2	72	16	1	22

Cultures from the spot enlargements induced in both these experiments contained Alternaria in nearly every case.

The experiments of September 9 were carried on in the laboratory. From a well-kept orchard York Imperial branches with perfect leaves were selected and the cut ends placed in a flask of distilled water. The material used in all five experiments

was sterilized by treatment with alcohol and with mercuric-chlorid solution and the leaves were burned with a heated rod, as in previous experiments. In experiments 1 to 4 the leaves were sprayed with distilled water containing, respectively, spores of *Coniothyrium pirinum*, *Sphaeropsis malorum*, *Alternaria* sp. from cultures made from spot enlargements in experiment 4 of July 28, and *Coryneum foliicolum*. In experiment 5 the leaves were sprayed with sterile water only and considered as checks. All the branches were then placed under bell jars lined with wet filter paper. Under these conditions the leaves retained their color and turgor for 10 days and appeared to be as healthy as if they had been left on the tree. The results are given in Table VII.

TABLE VII.—*Results of inoculation experiments of September 9 on York Imperial apple branches.*

Experiment No.	Number of burned spots.	Number of enlarged spots.		Average width of enlargements.		Percentage of enlarged spots.	
		Sept. 13.	Sept. 15.	Sept. 13.	Sept. 15.	Sept. 13.	Sept. 15.
				Mm.	*Mm.*		
1	101	15	53	1	1	15	52
2	94	0	11	0	1	0	12
3	113	52	107	1	1.5	46	95
4	83	2	9	1	1.5	2	11
5	46	1	2	1	1	2	4

The enlargements in experiment 3 were slightly larger than those in the other experiments. Coniothyrium was fruiting on the original spots in experiment 1, but not on the enlargements.

Of 14 cultures from enlargements of experiment 1 made as in previous series of experiments, 6 developed the Alternaria, 1 developed Coniothyrium, 4 contained bacteria, and 3 were sterile. Of 11 from experiment 2, 5 developed bacteria, 1 contained Alternaria, and 5 were sterile. From experiment 4, out of a total of 8 cultures, 4 developed Alternaria, 3 contained bacteria, and 1 was sterile.

In the series of experiments begun on September 17, York Imperial apple branches were used, as in previous experiments. All were placed under bell jars in the laboratory. Experiments 1 and 2 were performed in the same manner as the corresponding numbers in the experiments of September 9. In experiment 3 the leaves were sprayed with distilled water containing spores of *Monochaetia mali*, and in experiment 4, the check, they were sprayed with sterile water. All leaves were sterilized, and spots were burned upon them, as in previous experiments. The results on September 23 are given in Table VIII.

TABLE VIII.—*Results of inoculation experiments of September 17 on York Imperial apple branches.*

Experiment No.	Number of burned spots.	Number of enlarged spots.	Average width of enlargements.	Percentage of enlarged spots.
			Mm.	
1	87	17	1	20
2	167	32	2	19
3	65	3	1	5
4	53	10	2	19

Out of a total of 18 cultures from the spot enlargements of experiment 2, 17 contained Alternaria and 1 developed bacteria. In experiment 1 Coniothyrium fruits appeared on every spot, but there was none on the enlargements. As before, the favorite place

for the pycnidia of this fungus to appear was at the very edge of the original spot. In experiment 2, 7 spots, and in experiment 4, 2 spots had enlargements 4 mm. in width, with tufts of Alternaria hyphæ on their surfaces. All the enlargements were strikingly zonate.

In the experiments of September 24 the leaves of York Imperial branches received the same treatment as in the experiments of September 17, both as to sterilization and spotting. In experiments 1 to 3 the leaves were sprayed with distilled water containing spores of Phomopsis, Sphaeropsis, and Alternaria, respectively, while in No. 4, the check, they were sprayed with sterile water only. All the branches were then placed under bell jars lined with wet filter paper. The results after five days are given in Table IX. No attempts at reisolation were made.

TABLE IX.—*Results of inoculation experiment of September 24 on York Imperial apple branches.*

Experiment No.	Number of burned spots.	Number of enlarged spots.	Average width of enlargements.	Percentage of enlarged spots.
			Mm.	
1	99	2	1	2
2	71	3	1	4
3	102	47	1	46
4	51	4	1	8

It will be noticed that, owing to the natural prevalence of Alternaria on the leaves, sterilization was quite difficult. From the writer's experience in making cultures from fruit, leaves, and twigs of apples grown in the South, he believes Alternaria to be the most widely distributed fungus on the apple in that section of the country. In order to make the amount of natural infection about the same in all cases, all branches used in a series of experiments were sterilized together so that each leaf received the same treatment.

TABLE X.—*Summary of results from all experiments in which sterilized leaves were used.*

Treatment.	Number of burned spots.	Number of enlarged spots.	Percentage of enlarged spots.
Sprayed with spores of—			
Coniothyrium	471	123	27
Sphaeropsis	332	46	14
Alternaria	864	536	62
Phomopsis	259	6	2
Coryneum	83	9	11
Monochaetia	65	3	5
Sprayed with sterile water	928	62	7

The enlargements induced in each series of experiments bore a remarkable resemblance to the enlarged leaf-spot or frog-eye disease of the South. As shown in Plate VII, figure 3, the zonate effect was quite pronounced. This, as in the natural frog-eye, was due to an alternation of gray and brown coloration. Sometimes, also, the zone extended completely around the original spot, and sometimes its center lay at the margin of a spot, as is the case with frog-eye disease in nature. Alternaria was the only fungus isolated with any degree of consistency from any of these zones.

In some of the series of experiments the branches were removed from under bell jars for an hour every other day and the leaves allowed to dry. In such cases there seemed to be a rough relation between the number of such periods and the number of zones.

In one orchard in Virginia the leaves of eight York Imperial apple trees had been badly spotted through injury by sprays. Later, these spots enlarged and became typical frog-eye spots. Cultures made from these enlargements with the same precautions as previously outlined developed Alternaria in practically every case.

CONCLUSIONS

From these experiments one is justified in concluding that under certain conditions *Alternaria mali* is able to enlarge dead spots of apple leaves and may be classed as a rather strong facultative parasite. *Coniothyrium pirinum* possesses but little power of enlarging dead areas and may be classed as a saprophyte or at best as a weak facultative parasite. *Coryneum foliicolum*, *Phyllosticta limitata*, *Monochaetia mali*, and *Phomopsis mali* are, in so far as apple leaves are concerned, purely saprophytic.

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PLATE VII

Fig. 1.—Apple leaves from Tennessee, showing typical spots of the frog-eye disease.

Fig. 2.—Spores of *Alternaria mali*, which is capable of enlarging dead spots on apple leaves.

Fig. 3.—York Imperial apple leaves, showing spots enlarged by *Alternaria mali*. The dead centers of the spots were produced by burning with a heated rod.


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LONGEVITY OF PYCNOSPORES OF THE CHESTNUT-BLIGHT FUNGUS IN SOIL

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INTRODUCTION

That "spore horns" of the blight fungus *Endothia parasitica* (Murr.) And. are dissipated by rains has been recognized since the first studies on the chestnut bark disease. That the pycnospores are washed down the tree and are responsible for reinfections has been especially noted by Collins (1912).[1] This fact was also emphasized by Metcalf and Collins (1911), as may be noted from the following quotation:

> When the spores of the fungus are present, especially in the form of threads, or "horns," they are readily washed down the branches and trunk by every rain, and are thus carried down to or toward the base of the tree.

During the winter and spring of the year 1913 investigation of the dissemination of the chestnut-blight fungus by the writers disclosed the fact that during every rain, even at seasons when spore horns were not produced, pycnospores in great numbers were washed down the trunks of diseased trees. (Heald, 1913b; Heald and Gardner, 1913a–b.) This naturally led to the question as to what became of these millions of pycnospores washed into the soil.

Several possibilities suggested themselves. One of these was that the spores might germinate in the soil water and thus be readily killed by desiccation or other unfavorable conditions. Another was that, though the spores would not germinate, their period of viability might be very short even in wet soil, or that possibly they might retain their viability until it was terminated by unfavorable conditions such as freezing or drying out of the soil.

On the other hand, it was possible that the spores might remain viable not only during the time that the soil was wet but that they might endure for extended periods such unfavorable conditions as those produced by freezing or desiccation.

As to the possibility of pycnospores germinating in soil water, the tests so far carried out with various soil extracts have yielded inconclusive results. Pycnospores will germinate in certain soil extracts and not in others, the percentage of germination ranging from 0 to 65.

Our tests on the longevity of pycnospores in sterile tap water may be cited here as having some bearing on their persistence in wet soil. The few tests made have shown that 61 to 93 per cent retain their viability for four weeks. Only one trial was made to determine their power to endure freezing in sterile tap water. A very large percentage, 96, survived a period of six days of freezing, and 10 per cent survived a second period of eight days of freezing. Further investigation along all of these lines is planned.

Regarding the resistance of pycnospores to desiccation in soil, more conclusive results have been obtained. The purpose of the tests recorded

[1] Bibliographic citations in parentheses refer to "Literature cited," p. 75.

in the following pages was to determine the resistance of pycnospores washed into the soil in the field to desiccation in this soil under two distinct conditions: First, in the undisturbed soil in the field during the intervals between rains; and, second, during a more prolonged period of desiccation in the laboratory.

All except three of the soil samples used in these tests were secured in the experimental plat near West Chester, Pa., at the bases of diseased chestnut trees bearing certain of the "pycnospore traps" used in the analysis of rain water washed down the trunks (Heald and Gardner, 1913a–b). The results of these analyses proved that few, if any, ascospores were present in the water washed down the trees into the soil, even late in the spring after ascospore expulsion was in progress. From these data and from the time of appearance of the colonies in our cultures it is certain that the spores with which we were dealing were pycnospores and not ascospores (Heald, 1913a).

TESTS OF LONGEVITY UNDER FIELD CONDITIONS

The first samples of soil in each series used in determining the power of resistance of the pycnospores to desiccation in soil under field conditions were taken as soon as possible after the cessation of a rain. The litter and immediate surface soil were scraped away from a small area at the base of the tree, and with a sterile knife or spoon a sample of the soil thus exposed and immediately adjacent to the base of the trunk was removed to a sterile tin receptacle for transport to the laboratory.

The dilution method with poured-plate cultures was used in ascertaining the number of viable pycnospores per gram of soil. Using sterile utensils, 1 gram of the soil was weighed out and placed in a flask containing 99 c. c. of sterile water. By crushing the soil lumps with a flamed glass rod and by agitation of the flask a thoroughly uniform suspension was obtained. From this suspension 1 c. c. was transferred with a sterile pipette to a second flask containing 99 c. c. of sterile water, and of this second dilution measured quantities (1 c. c. and fractions thereof) were placed in sterile Petri dishes and plated out in standard 3 per cent dextrose agar +10. From the number of colonies of the blight fungus developing in these cultures an estimate was obtained of the number of viable pycnospores present in the gram of soil tested.

In securing the second or third samples of soil from each location in the field the soil was taken from a point as near as possible to the exact source of the first sample and from a similar position relative to the tree. The method used in testing these subsequent samples was identical with that previously outlined, no allowance being made for loss of weight due to drying. In making a second or third test it is evident that a different individual portion of soil must be used in which the original number of spores contained would probably by no means coincide with the spore content of the previous sample from that location. Taking this fact into consideration, the discrepancies in the figures are only such as might be expected.

The period of desiccation in these field determinations was necessarily limited by the recurrence of rain, so that in series A, B, and C only one subsequent test was obtained for each location. In series D, however, a rather extended period of dry weather permitted tests to be made after relatively longer periods of desiccation. The results obtained are presented in Table I.

TABLE I.—*Longevity tests of pycnospores in soil in the field in 1913.*

[Samples taken from bases of diseased chestnut trees bearing pycnospore traps.]

SERIES A.

Trap No. and soil sample No.	Rainfall. Date.	Rainfall. Inches.	Date of collection.	Date of cultures.	Period of drying.	Condition of soil when tested.	Number of viable spores per gram of soil.
					Days		
Trap I:							
Sample 1	Mar. 20	1.64	Mar. 21	Mar. 21	1	Wet from rain	1,140,000
Sample 3			Mar. 24	Mar. 24	4	Nearly air-dry	47,072
Trap III:							
Sample 2	do.	1.64	Mar. 21	Mar. 21	1	Wet from rain	527,724
Sample 4			Mar. 24	Mar. 24	4	Nearly air-dry	21,509

SERIES B.

Trap I:							
Sample 5	Apr. 4	.56	Apr. 5	Apr. 5	½	Wet from rain	3,604,000
Sample 7			Apr. 7	Apr. 7	2½	Damp	7,128,000
Trap III:							
Sample 6	do.	.56	Apr. 5	Apr. 5	½	Wet from rain	4,410,000
Sample 8			Apr. 7	Apr. 7	2½	Damp	1,998,000

SERIES C.

Trap I:							
Sample 9	Apr. 10–16.	4.06	Apr. 18	Apr. 19	2½	Damp from rain	6,570,000
Sample 11			Apr. 21	Apr. 22	5½	Air-dry	1,640,000
Trap III:							
Sample 10	do.	4.06	Apr. 18	Apr. 19	2½	Damp from rain	2,412,666
Sample 12			Apr. 21	Apr. 22	5½	Air-dry	288,000

SERIES D.

Trap I:							
Sample 13	Apr. 27–28.	2.43	Apr. 28	Apr. 29	0	Very wet	1,209,500
Sample 17			May 5	May 6	6	Air-dry	2,723,333
Sample 21	Apr. 29	.11	May 12	May 13	13	Loose, air-dry surface soil exposed to sun.	1,077,333
Trap III:							
Sample 14	Apr. 27–28.	2.43	Apr. 28	Apr. 29	0	Very wet	2,948,000
Sample 18			May 5	May 6	6	Air-dry	86,000
Sample 22	Apr. 29	.11	May 12	May 13	13	Loose, air-dry surface soil partly shaded.	84,000
Trap IV:							
Sample 15	Apr. 27–28.	2.43	Apr. 30	May 1	1	Wet from rain	3,336,000
Sample 19			May 5	May 6	6	Air-dry	3,792,000
Sample 23	Apr. 29	.11	May 12	May 13	13	Loose, air-dry surface soil exposed to sun.	2,412,333

TABLE I.—*Longevity tests of pycnospores in soil in the field in 1913*—Continued.

Trap No. and soil sample No.	Rainfall. Date.	Rainfall. Inches.	Date of collection.	Date of cultures.	Period of drying.	Condition of soil when tested.	Number of viable spores per gram of soil.
					Days		
Trap VI:							
Sample 16.	Apr. 27–28.	2.43	Apr. 30	May 1	1	Wet from rain..	490,000
Sample 20.			May 5	May 6	6	Air-dry.....	1,100,000
Sample 24.	Apr. 29	.11	May 12	May 13	13	Loose, air-dry surface soil exposed to sun.	368,000

TESTS OF LONGEVITY UNDER LABORATORY CONDITIONS

For use in determining the longevity of pycnospores dried in soil and stored in the culture room in the laboratory to secure longer periods of desiccation, samples were collected in much the same manner as has been described for the field tests. A much larger quantity of soil was taken from the bases of diseased trees after a rain and transported to the laboratory in sterile tin containers. During storage in the culture room the tin covers were replaced by layers of absorbent cotton held in place by rubber bands. Thus, ample opportunity was afforded for thorough drying of the soil.

These samples were tested as soon as possible after collection while the soil was still wet and at convenient intervals thereafter during the period of storage to ascertain the number of viable pycnospores to the gram. With some exceptions the method used in the tests was identical with that already described for the field tests. Previous to each test, however, the soil in each container was thoroughly shaken to secure as uniform a mixture as possible. As in the previous work, no allowance was made for loss of weight due to evaporation of the soil moisture. Most of this loss occurred during the first period of drying, the soil being practically air-dry and readily reducible to dust at the end of one week. By numerous trials the loss in weight due to air-drying was found to represent an average decrease of 35 to 40 per cent of the weight at the time the first test was made. A factor of 1.66⅔ might be applied to the figures given in the first or control analysis of each sample to compensate for this error.

In all of the cultures made previous to July 30, 3 per cent dextrose agar + 10 was used as the medium. In the cultures made on July 30, August 8, and August 26 chestnut-bark agar made from diseased bark was employed; in the final tests chestnut-bark agar made from healthy bark was employed. Furthermore, relatively larger portions of inoculum were used in the final tests (September 25), 1 c. c. being transferred from the first suspension to a flask containing only 9 c. c. of sterile water from which 1 c c. and fractions thereof were plated out. The results obtained are presented in Table II.

TABLE II.—*Longevity tests of pycnospores in soil stored in culture room in 1913.*

SERIES E.

[Rainfall: Date, April 27 and 28; amount, 2.43 inches. Date of collection of soil samples, April 28. Source of soil samples: No. 13. Base of sprout bearing pycnospore trap No. I. No. 14. Base of tree bearing pycnospore trap No. III.]

Date of test.	Number of days of drying.	Number of viable spores per gram of soil.	
		Soil sample No. 13.	Soil sample No. 14.
Apr. 29	0	1,209,500	2,948,000
May 8	9	282,000	240,000
May 16	17	42,000	446,000
May 23	24	62,000	217,142
May 31	32	0	280,000
June 6	38	205,333	270,000
June 12	44	116,875	504,000
June 19	51	12,666	216,000
June 27	59	54,000	41,428
July 3	65	11,428	1,272,000
July 11	73	0	30,526
July 20	82	25,333	31,356
July 30	92	20,571	0
Aug. 8	101	0	0
Aug. 26	119	0	17,857
Sept. 25	149	0	0

SERIES F.

[Rainfall: Date, Apr. 27, 28, and 29; amount, 2.54 inches. Date of collection of soil samples, Apr. 30. Source of soil samples: No. 15. Base of tree bearing pycnospore trap No. IV. No. 16. Base of tree bearing pycnospore trap No. VI.]

Date of test.	Number of days of drying.	Number of viable spores per gram of soil.	
		Soil sample No. 15.	Soil sample No. 16.
May 1	0	3,336,000	490,000
May 8	7	820,000	91,875
May 16	15	532,000	53,333
May 23	22	51,428	144,000
May 31	30	204,054	68,571
June 6	36	96,000	95,143
June 12	42	62,666	43,333
June 19	49	92,000	70,000
June 27	57	63,000	(?)
July 3	63	42,857	0
July 11	71	0	14,000
July 20	80	18,636	0
July 30	90	11,714	0
Aug. 8	99	0	0
Aug. 26	117	0	0
Sept. 25	147	0	0

TABLE II.—*Longevity tests of pycnospores in soil stored in culture room in 1913*—Contd.

SERIES G.

[Rainfall: Date, May 16 and 17; amount, 0.74 inches. Date of collection of soil samples, May 19. Source of soil samples: Orchard of grafted Paragon trees, Martic Forge, Pa.[1] No. 17. Base of tree bearing ascospore trap No. 44. No. 18. Base of tree bearing ascospore trap No. 43. No. 19. Base of tree bearing ascospore trap No. 43.]

Date of test.	Number of days of drying.	Number of viable spores per gram of soil.		
		Soil sample No. 17.	Soil sample No. 18.	Soil sample No. 19.
May 20	0	4,106,666	1,196,000	8,890,000
May 27	7	3,808,000	925,000	1,840,000
June 4	15	2,318,666	630,000	4,966,000
June 12	23	1,351,250	530,000	4,156,666
June 19	30	816,000	94,285	3,192,000
June 27	38	920,000	116,000	2,240,000
July 3	44	468,000	(?)	1,662,727
July 11	52	733,333	30,000	972,571
July 20	61	204,545	0	90,000
July 30	71	68,148	46,000	59,259
Aug. 8	80	0	0	0
Aug. 26	98	0	0	5,000
Sept. 25	128	0	0	0

[1] Collected by Mr. C. E. Taylor, formerly in the employ of the Pennsylvania Chestnut Tree Blight Commission.

SERIES H.

[Rainfall: Date, May 16 and 17; amount, 0.61 inch. Date of collection of soil samples, May 19. Source of soil samples: No. 20. Base of tree bearing pycnospore trap No. III. No. 21. Base of tree bearing pycnospore trap No. VI.]

Date of test.	Number of days of drying.	Number of viable spores per gram of soil.	
		Soil sample No. 20.	Soil sample No. 21.
May 21	0	2,832,000	2,640,000
May 27	6	750,000	1,063,333
June 4	14	364,000	571,428
June 12	22	100,800	294,853
June 19	29	26,666	150,857
June 27	37	170,500	115,142
July 3	43	68,181	140,000
July 11	51	21,714	82,000
July 20	60	15,000	21,176
July 30	70	177,777	25,925
Aug. 8	79	12,413	0
Aug. 28	97	0	0
Sept. 25	127	0	0

TABLE II.—*Longevity tests of pycnospores in soil stored in culture room in 1913*—Contd.

SERIES K.

[Rainfall: Date, May 21, 22, 23, and 24; amount, 3.24 inches. Date of collection of soil samples, May 26. Source of soil samples: No. 22. Base of tree bearing pycnospore trap No. I. No. 23. Base of tree bearing pycnospore trap No. IV. No. 24. Base of tree bearing pycnospore trap No. VI.]

Date of test.	Number of days of drying.	Number of viable spores per gram of soil.		
		Soil sample No. 22.	Soil sample No. 23.	Soil sample No. 24.
May 27	0	3,760,000	4,480,000	2,270,333
June 4	8	1,300,000	1,530,000	1,050,000
June 12	16	38,571	578,000	270,000
June 19	23	52,000	160,000	82,285
June 27	31	1,611,428	90,000	206,666
July 3	37	553,714	322,000	92,571
July 11	45	0	145,454	0
July 20	54	62,000	70,454	0
July 30	64	28,000	0	11,000
Aug. 8	73	0	0	0
Aug. 26	91	0	0	5,000
Sept. 25	121	0	0	0

SUMMARY.

No. of soil sample.	Number of viable spores per gram of soil before drying.	Number of days spores remained viable (longevity limit).	Number of viable spores per gram of soil in last test showing presence of viable spores.
13	1,209,500	92	20,571
14	2,948,000	119	17,857
15	3,336,000	90	11,714
16	490,000	71	14,000
17	4,106,666	71	68,148
18	1,196,000	71	46,000
19	8,890,000	98	5,000
20	2,832,000	79	12,413
21	2,640,000	70	23,925
22	3,760,000	64	28,000
23	4,480,000	54	70,454
24	2,270,333	91	5,000

CONCLUSIONS

The results obtained in these two sets of tests lead to several conclusions. The field tests show that the pycnospores are to a considerable degree resistant to desiccation in soil in the field and that a large number may retain their viability during a period of 2 to 13 days of dry weather.

In the indoor tests, where the period of drying could be prolonged at will, more conclusive results were secured. In each sample it is seen that, with some irregularities, there is a gradual decrease in the number of viable spores as the period of desiccation is prolonged. The irregularities in the figures are due partly to the fact that from the standpoint of spore content it was impossible to secure a perfectly uniform mixture in the soil sample.

It is evident that in every case except one a large number of spores survived two months of desiccation and that in 5 out of the 12 samples not all of the spores had succumbed after three months of drying. The longevity limit varies from 54 to 119 days, the average being 81 days. At the end of periods of desiccation ranging from 121 days for some samples to 149 days for others no viable spores were found. The conclusiveness of these final tests is enhanced by the fact that relatively larger quantities of inoculative material were used in the cultures. (See summary of Table II.)

These results suggest that viable pycnospores are constantly present in the soil beneath infected trees, since each succeeding rain replenishes the supply. Under normal conditions there would hardly be a period of drought sufficiently extended to destroy all of the enormous number washed into the soil.

The fact that a large percentage of the pycnospores which are washed into the soil withstand two to three months of drying has an interesting bearing upon certain phases of the problem of dissemination of the chestnut bark disease and affords at least three possibilities for conjecture.

First, it shows that pycnospores will resist the degree of drying necessary to reduce the soil to a condition in which it might be easily pulverized and blown about as dust. This presents the possibility of wind dissemination of pycnospores dried on soil particles. Such a possibility has been suggested by Metcalf and Collins (1911), and Collins (1912). However, this contingency is rather improbable under natural forest conditions, since the soil beneath the trees is more or less protected and dries much less rapidly than in the open. On the other hand, there would be a fair probability of wind transport of pycnospores in blight-infected nurseries without ground cover or from more isolated trees, especially if the soil is exposed or the ground cover is sparse.

In the second place, a means is presented whereby pycnospores might be transported in mud dried on insects, on the feet of birds, squirrels, and other animals, and even on the shoes of man. Such might be the explanation of the presence of pycnospores found on one of the two juncos tested during the study of birds as carriers of the chestnut blight fungus (Heald and Studhalter, 1913), since the junco obtains nearly all of its food from the ground.

Finally, the results of these tests present the possibility of the transportation of pycnospores in the soil adhering to the stems and roots of chestnut nursery stock during shipment. Such nursery stock might be

uninfected and hence pass inspection as free from disease, the spores having been washed into the soil from diseased parts pruned from the specimens before shipment or from diseased plants which were adjacent to the healthy specimens in the nursery. Under such conditions it is evident that large numbers of pycnospores might retain their viability during long periods of shipment.

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